STRUCTURE-FUNCTION RELATIONSHIPS OF THE MAJOR NEUROTOXIN FROM THE SEA ANEMONE *STICHODACTYLA HELIANTHUS* WITH A NEW SODIUM CHANNEL RECEPTOR SITE

By

MICHAEL WILLIAM PENNINGTON

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

This dissertation is dedicated
to my mother and father, to Kim Lassiter
and to the memory of Dr. Carolyn Bourne.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Dr. Ben M. Dunn, and Dr. William R. Kem as well as the rest of the members of my committee for their advice and assistance in the preparation of this dissertation. In addition, I am indebted to Dr. Jan Pohl, whose valuable insight into the characterization of peptides as well as helpful suggestions, gave me a role model to follow. Special thanks to all those who learned to work around my rather bizzare schedule and still remained my friends. Finally, I would like to thank the following people for their help and/or support: Benne Parten, Jeff Weidner, Mark Carter, Rohit Cariappa, Mike Campa, Dave Marriott, Susie Pennington-Hammersley, John Hamilothoris, Kevin Krajniak, Tom Nutter, and Dr. Peter Anderson.

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ABBREVIATIONS

AaH	*Androctonus austrialis Hector*
AcOH	acetic acid
Af	*Anthopleura fuscovirdis*
As	*Anemonia sulcata*
Asn_{11}	Asn analog of ShN at position 11
Asn_6	Asn analog of ShN at position 6
Asn_7	Asn analog of ShN at position 7
ATP	adenosine triphosphate
Ax	*Anthopleura xanthogrammical*
B_{max}	maximum theoretical binding sites
Bol	*Bolecera tuediae*
BSA	bovine serum albumin
[^{13}C] NMR	carbon thirteen nuclear magnetic resonance
BTX	batractotoxin
C_{18}	octadecyl silica
CD	circular dichroism
Ci	curie
Condyl III	*Condylactus gigantea*
cpm	counts per minute
CsE	*Centruroides sculpturatus Ewing*
Css	*Centruroides suffusus suffusus*

C-terminal	carboxy-terminal
DDT	1,1,1 trichloro-2,2 bis (*p*-chlorophenyl)ethane
°C	degrees Celsius
dH_2O	distilled water
DTNB	*5,5′*-dithiobis(2-nitrobenzoate)
ED_{50}	effective dose for 50% effect
EDTA	ethylenediaminetetra-acetic acid
Fab	antigen binding domain of digested antibody
fmol	femptomoles (10^{-15} moles)
Gln_8	Gln analog to ShN I at postion 8
GSH	glutathione, reduced form
GSSG	glutathione, oxidized form
h	hours
HCl	hydrochloric acid
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HF	anhydrous Hydrogen Floride
Hm	*Heteractis macrodactylis*
Hp	*Heteractis paumotensis*
HPLC	high pressure liquid chromatography
I_5A	insect toxin isolated from *Buthus eupeus*
$K_{0.5}$	displacement of 50% of labeled compound

K_D	dissociation constant
l	liter
LD_{50}	lethal dose for 50% of the animals
Lqq	*Lieurus quinquestriatus quinquestriatus*
M	moles per liter
μg	microgram (10^{-6} g)
μl	microliter (10^{-6} l)
μM	micromolar (10^{-6} moles/l)
μmol	micromole (10^{-6} mole)
mg	milligram (10^{-3} g)
msec	millisecond
min	minute
Na channel	sodium channel
$^{22}Na^{+}$	radioactive isotope of sodium
$NacK_4$	N-acetyl Lys analog of ShN I at position 4
nM	nanomolar (10^{-9} moles/l)
nmol	nanomole (10^{-9} mole)
NMR	nuclear magnetic resonance
N-terminal	amino-terminal
PBS	phosphate buffered saline
pH	-log $[H^{+}]$
pK_a	dissociation constant of a proton
pmol	picomole (10^{-12} moles)
$[^1H]$ NMR	proton nuclear magnetic resonance

PTH	phenylthiohydantoin
RIA	radioimmunoassay
Sg	*Stichodactyla gigantea*
Sh	*Stichodactyla helianthus*
STX	saxitoxin
TFA	trifluoroacetic acid
TiTxτ	*Tityus serulatus* toxin τ
*Tityus*toxin	*Tityus serulatus* toxin
Tris HCl	tris(hydroxymethyl)aminomethane hydrochloride
Tris OAc	tris(hydroxymethyl)aminomethane acetate
[^{3}H]-BTX-benzoate	tritiated derivative of batrachotoxin
[^{3}H]-STX	tritiated deriative of saxitoxin
[^{3}H]-TTX	tritiated derivative of tetrodotoxin
TTX	tetrodotoxin
2D-NMR	two-dimensional nuclear magnetic resonance
Tyr_1	analog incorporating Tyr at first position of ShN I
V_o	void volume

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By

MICHAEL WILLIAM PENNINGTON

December, 1988

Chairman: B.M. Dunn, Ph.D.
Major Department: Biochemistry and Molecular Biology

We have determined that ShN I, a 48-residue type 2 sea anemone toxin, delays the inactivation of the Na channel in lobster olfactory somas. The effects of this toxin are similar to those observed for the α-scorpion toxins and type 1 sea anemone toxins, yet ShN I is structurally distinct from the type 1 toxins. Using a polyclonal antibody prepared against ShN I, no crossreactivity was observed to exist between the type 1 and type 2 anemone toxins.

The receptor for ShN I was identified in vesicle preparations of neuronal tissues from both crustaceans and mammals; however, the K_D value for the former is more than 1,000 fold lower than for the later. The binding of [^{125}I]-ShN I to this receptor was determined to be unaffected by *Anemonia*

sulcata II, depolarization of the membrane, or veratridine. ShN I was unable to displace [^{125}I]-*Androctonus australialis Hector* II, whereas unlabeled AaH II and As II displaced the labeled scorpion toxin from rat brain synaptosomes. This is the first characterization of a new Na channel receptor site which specifically binds type 2 anemone toxins.

To study the interactions that specific amino acid residues of ShN I have with this receptor, we developed a strategy using solid phase peptide synthesis. Prior to the synthesis of analogs to ShN I, we assembled the native ShN I sequence and reoxidized the three intramolecular disulfide bonds. Chemical, physical, and pharmacological characterization of the purified synthetic ShN I showed it to be indistinguishable from the natural toxin.

Monosubstituted analogs of ShN I were synthesized to probe the high density of charged residues localized in the N-terminal region of the molecule. Following oxidation of the disulfide bonds, purification and characterization, these analogs were tested in vivo on fiddler crabs and in vitro on vesicles prepared from crab walking leg nerves. Both of these studies implicated the tri-anionic region of Asp_6-Asp_7-Glu_8 as essential for activity. Substitutions at the positions of Lys_4 and Asp_{11} resulted in analogs with markedly reduced activity and increased $K_{0.5}$ values. Substitution of tyrosine for the N-terminal residue had almost no effect on either of these values.

CHAPTER I

INTRODUCTION

Historical Elucidation of the Sodium Channel

Action potentials are the rapidly propagating electrical messages that travel along axons of the nervous system and over the surface of some muscle and glandular cells. In axons, they are characterized as being short-lived signals, which travel at a constant velocity and maintain a constant amplitude (Hille, 1984).

The axon can be considered as a cylinder of axoplasm surrounded by a continuous surface membrane. The membrane is essential in that it serves as a barrier for the development of an ionic gradient. Bernstein (1902, 1912) was the first to develop a hypothesis considering the membrane to be central in the propagation of an action potential. The membrane potential is defined as the intracellular potential minus the extracellular charge. Measurement of the membrane potential can be made with a special glass microelectrode filled with a stock solution of 3 M KCl. This microelectrode can be carefully passed through the cell membrane of a neuron to measure the potential (Hille, 1984).

Cole and Curtis (1939) were the first to record the changes in the electrical conductivity of the membrane from the squid

(*Loligo forbesi*) giant axon. They observed that the membrane undergoes a large change in conductance which occurs in the same time scale as the electrical change. This experiment supported the theory of an increase in ionic permeability but did not determine the ions involved.

Hodgkin and Huxley (1939, 1945) and Curtis and Cole (1940, 1942) were the first to measure the full action potential of an axon using an intracellular recording microelectrode. Shown in Figure 1:1, an action potential develops following an electrical stimulus to the neuron. The action potential develops approximately 0.5 msec after the stimulus at which time the membrane is observed to depolarize. As the action potential propagates down the length of the axon, the cell then repolarizes. This response is characteristic of all electrically excitable tissues and it is always an all-or-none response (Hille, 1984).

An unexpected result observed in these classical experiments was that the action potential overshot zero and reversed in sign to positive membrane potential values (Hodgkin and Huxley, 1939, 1945; Cole and Curtis, 1940, 1942). This positive overshoot was not understood until Hodgkin and Katz tested their sodium permeability theory (Hodgkin and Katz, 1949). This theory incorporated previously reported results that the membrane potential at resting values in squid giant axon was primarily

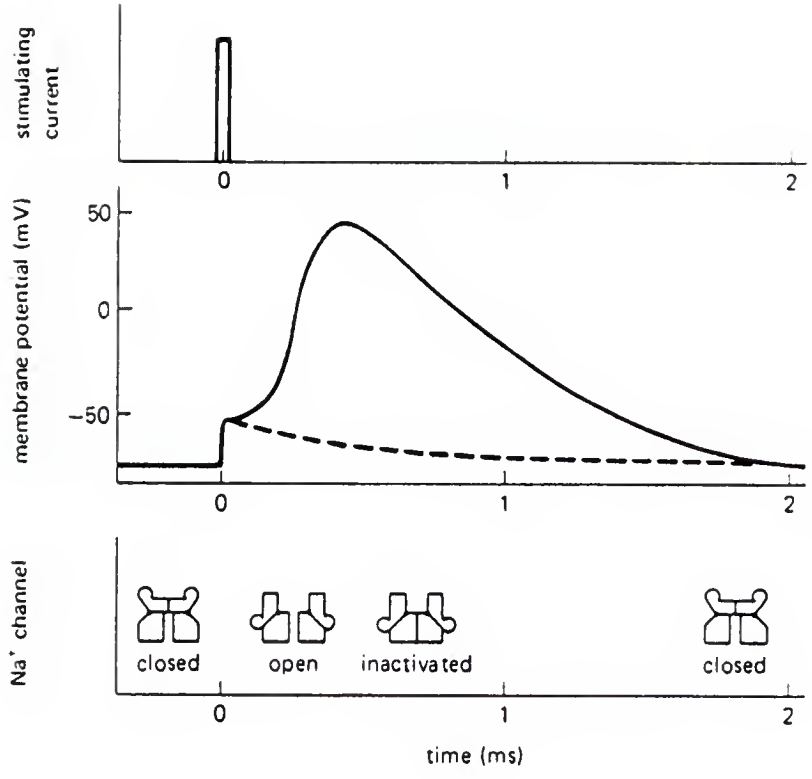

Figure 1:1: Action potential recording following a brief pulse of current that partially depolarizes the membrane. In the middle graph, the response of the Na channel opening and subsequent inactivation are shown in the solid line. The broken line shows the membrane response in the absence Na channels. The bottom illustration shows the sequence of resting, open and closed (inactivated) states following a stimulus. Taken from Alberts et al., 1983).

due to selective potassium permeability (Goldman, 1943). Following a stimulus, the positive overshoot of an action potential resulted from the inward passage of Na^+ into the axon through the membrane, causing a change in the resting membrane potential to values near that of the equilibrium potential of Na^+. Decreasing the Na^+ concentration in the extracellular medium with nonpermeable salts such as choline chloride or other small molecules such as glucose decreased the amplitude of the action potential (Hodgkin and Katz, 1949). These results determined Na^+ to be the ion responsible for the permeability changes observed.

Following these experiments, radioactive $^{22}Na^+$ was used to investigate the movement of Na^+ across the membrane in squid giant axon. Keynes and Lewis (1951), using axoplasm activation analysis, determined that a single impulse resulted in the net movement of 20,000 Na^+ ions through 1 μm^2. This value was shown to be within experimental error of the number of ions necessary to result in the 120 mV voltage change associated with an action potential (Huxley, 1964).

The voltage clamp technique was developed at this time in several labs (Marmont, 1949; Cole, 1949; and Hodgkin et al., 1949, 1952). This technique maintains a constant potential across the membrane with a feedback amplifier, allowing changes in the ionic currents to be measured. Using this technique, Hodgkin and Huxley (1952) were able to show that the action potential in squid giant axon results from the time and voltage-

dependent increases in the axonal membrane permeability to Na^+ and K^+. These experiments showed that during a maintained depolarization, the Na^+ permeability initially increases for a few msec, then returns to resting values. The K^+ permeability lags behind that of Na^+ and is rising to the maximum value as the Na^+ permeability returns to resting values. As the resting membrane potential is reachieved, the K^+ permeability decreases to resting values. The Hodgkin and Huxley experiments (1952) showed that these changes can be separated into two voltage-dependent processes: activation, which controls the rate and voltage dependence of the Na^+ permeability increase following a depolarization, and inactivation, which controls the rate and voltage-dependence of the subsequent return of the Na^+ permeability to the resting level during a maintained depolarization. This gives rise to the simplistic model system which consists of three states or groups of states: resting, activated (open), and inactivated (closed). The resting and the inactivated states are both nonconducting processes which differ from each other in that the inactivated state is refractory to further stimulus until repolarization of the membrane.

Pharmacological studies using TTX, a natural product isolated from puffer fish of the order Tetraodontiformes (Halstead, 1978), resolved the question concerning the actual mechanism by which ions pass through the membrane. In voltage clamp experiments, Narahashi et al. (1964) showed that TTX blocks the Na current without affecting the K current on lobster giant

axon. The toxin exerted these effects at nanomolar concentrations. Similar studies with STX, a natural product isolated from dinoflagellates of the genus *Gonyaulax* responsible for the "red tide" (Taylor and Selinger, 1979), showed identical effects when tested on the electroplax of *Electrophorus electricus* (Nakamura et al., 1965), lobster giant axon (Narahashi et al., 1967), and frog node of Ranvier (Hille, 1967, 1968a, 1968b). The discovery of a specific K^+ current blocking compound, tetraethylammonium, by Tasahi and Hagiwara, (1957) provided further evidence for the existence of separate transporters (channels) for these two ions.

The size of the pore responsible for the passage of Na^+ was estimated by Hille (1971). In this study, the ability of several nonmethylated organic cations such as ammonium, guanidinium, hydroxylammonium, and hydrazinium to pass through the axonal membrane was measured. The results of these experiments determined a minimum pore size of 3 Å by 5 Å.

The pore mechanism for ionic transport was supported by determination of the capacity of the channel. Using the voltage clamp technique on squid giant axon to measure Na current and a [^{3}H]-TTX derivative to estimate Na channel density, a conductance of 2.5-8.6 pS was measured (Levinson and Meves, 1975; Almers and Levinson, 1975). Other methods including patch clamping, a technique which measures the ionic currents through a small isolated patch of membrane (Sigworth and Neher, 1980; Nagy et al., 1983), and Fourier transform analysis of Na current

fluctuations (Conti et al., 1975, 1976) determined values between 4.1-18 pS. These values would result in ion transport rates in excess of 10^7 ions/sec, which exceed those measured for small antibiotic ion carriers such as valinomycin and gramicidin with measured rates of 10^4 ions/sec (Stark et al., 1971).

Selectivity for Na^+ ions is another important property of the Na channel. Of the rare earth ions, Na^+ is the most permeable, with K^+ being only 8% as permeable as Na^+. The other rare earth ions, Rb^+ and Cs^+, are even less permeable than K^+ (Chandler and Meeves, 1965; Moore et al., 1966). The selectivity of the Na channel was postulated to reside inside the pore of the channel as an ion selectivity filter which would block the passage of molecules with dimensions greater than 3 Å by 5 Å (Hille, 1971, 1972).

Identification of Toxin Binding Sites

Following the electrophysiological dissection of the action potential, the discovery of a Na^+ specific channel was not fully accepted until the discovery that TTX specifically blocks Na current reported by Narahashi et al., (1964). The pharmacological and biochemical characterization of the Na channel was initiated with the compounds TTX and STX, and greatly advanced through the discovery of other toxins which bind with high affinity and specificity to the Na channel. These toxins which act on excitable tissues have been given the name neurotoxins. The number of neurotoxin binding sites on the

Na channel continues to increase with the discovery of new toxic compounds. The neurotoxins have been classified according to the effects which they exert on the Na channel.

The first class of toxins consist of the guanidinium toxins STX and TTX as well as the μ-conotoxins, which are a series of polypeptides isolated from the marine snail *Conus geographus* (Sato et al., 1983; Cruz et al., 1985). The structures for the guanidinium toxins are shown in Figure 1:2, and the sequences of the μ-conotoxins are shown in Figure 1:3. The guanidinium toxins act in very low concentrations (nM) at a small number of discrete sites on the membrane. Initial attempts at quantitating the number of sites was based upon bioassay of the bathing medium following blockage of the action potential in lobster giant axon. The results of the experiments suggested an upper limit of 13 sites/μm^2 of axonal membrane (Moore et al., 1967). Second generation experiments to determine the number of binding sites used radiolabeled derivatives of both TTX and STX. Using tritium exchange, exchangeable protons were replaced with tritium to yield [^{3}H]-TTX and [^{3}H]-STX derivatives with specific activities of 10 Ci/mmol (Ritchie et al., 1976). Binding studies with these radiolabeled derivatives determined saturable binding components with K_D values from 1 to 10 nM for all the tissues studied (Ritchie et al., 1976; Ritchie and Rogart, 1977 a,b; Catterall and Morrow, 1978; Strichartz et al., 1979). More recently, studies on Na channel in cardiac tissue have shown at least two types of Na channel: one with high affinity for STX

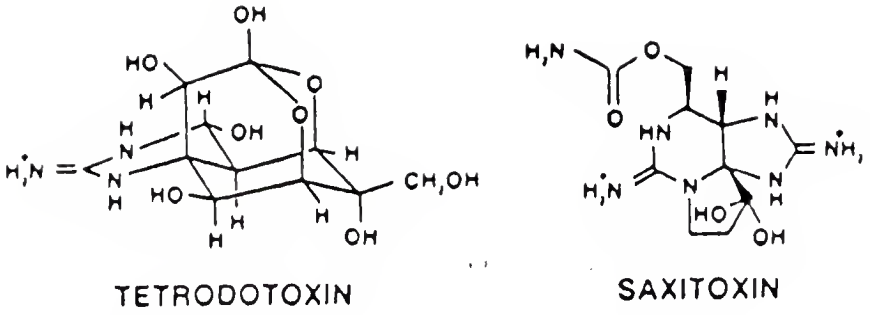

Figure 1:2: Molecular structures of the guanidinium toxins, Tetrodotoxin (TTX) and Saxitoxin (STX). Taken from Hille, 1984.

GIIIA	R·D·C·C·T·Hyp·Hyp·K·K·C·K·D·R·Q·C·K·Hyp·Q·R·C·C·A-NH_2
[Pro^6]GIIIA	R·D·C·C·T·P·Hyp·K·K·C·K·D·R·Q·C·K·Hyp·Q·R·C·C·A-NH_2
[Pro^7]GIIIA	R·D·C·C·T·Hyp·P·K·K·C·K·D·R·Q·C·R·Hyp·Q·R·C·C·A-NH_2
GIIIB	R·D·C·C·T·Hyp·Hyp·R·K·C·K·D·R·R·C·K·Hyp·M·K·C·C·A-NH_2
[Pro^6]GIIIB	R·D·C·C·T·P·Hyp·R·K·C·K·D·R·R·C·K·Hyp·M·K·C·C·A-NH_2
[Pro^7]GIIIB	R·D·C·C·T·Hyp·P·R·K·C·K·D·R·R·C·K·Hyp·M·K·C·C·A-NH_2
GIIIC	R·D·C·C·T·Hyp·Hyp·K·K·C·K·D·R·R·C·K·Hyp·L·K·C·C·A-NH_2

Figure 1:3. Amino acid sequences of the μ-conotoxins. Taken from Cruz et al. (1985).

(Renaud et al., 1986), and a second type which has a significantly higher K_D value for STX and TTX (1 μM) (Catterall and Coppersmith, 1981a, 1981b).

The μ-conotoxins have been shown to have no effect on either neuronal (both rat and lobster) or cardiac (rat) forms of the Na channel. Competition assays have shown that μ-conotoxins displace [^{3}H]-STX from rat skeletal muscle membranes and electroplax membranes. The K_D values for the μ-conotoxins GIIIA and GIIIB were determined to be 50 nM for electroplax membranes, K_D values of 25 and 140 nM, respectively, were determined for rat skeletal muscle membranes (Moczydlowski et al., 1986). The μ-conotoxin GII was determined to have a $K_{0.5}$ values of 35 nM on rat T-tubular membranes and 60 nM on rat skeletal muscle homogenates (Ohizumi et al., 1986). These results indicate a tissue specific distribution of different Na channel types.

The binding site for the guanidinium toxins has been proposed to reside at or near the ion selectivity filter (Kao and Nishiyama, 1965; Henderson et al., 1974; Hille, 1975). The ion selectivity filter had been shown to contain an essential carboxyl group with a pK_a value of 5.4. This acidic group must be ionized for channel function (Woodhull, 1973). Similarly, TTX and STX binding are completely inhibited by protonation of an acidic group with a pK_a value of 5.4 (Henderson et al., 1973; Henderson et al., 1974; Balerna et al., 1975). Experiments where this carboxyl group was modified through carbodiimide mediated amidation (Shranger and Porfera, 1973) or alkylated

with trialkyloxonium salts (Reed and Raftery, 1976; Baker and Rubinson, 1975, 1976) determined that the modified Na channel was unable to bind TTX. Furthermore, following treatment with trimethyloxonium, the Na channel is irreversibly modified; however, the ability to generate action potentials is not abolished. It was decreased to 40% of the original value (Sigworth and Spalding, 1980). This implies that the reactive carboxyl group is close to the pore, but it does not determine ionic selectivity. Following modification, an unmodified carboxyl group still exists within the pore which can be protonated with acid solutions to block Na channel function. Therefore, at least two carboxyl groups have been implicated in the ion selectivity filter. When modification reactions were performed in the presence of saturating amounts of TTX, alkylation of these carboxyl groups was blocked (Spalding, 1980).

The guanidinium toxins are believed to bind to the Na channel receptor through the interaction of the charged guanidinium group with a carboxyl on the extracellular face of the transmembrane pore (Kao and Nishiyama, 1965; Hille, 1968a), which may possibly be part of the ion selectivity filter (Hille, 1971). These molecules function by blocking the Na current directly without altering the properties of activation or inactivation (Narahashi et al., 1964; Nakamura et al., 1965; Hille, 1968). Binding of these compounds is not affected by voltage (Almers and Levinson, 1975; Catterall et al., 1979) or

by other toxins which bind and alter different properties of the channel (for review see Catterall, 1980). In tissues with high affinity TTX and STX binding sites, no interactions between binding and gating have been found (Catterall, 1980; Ulbricht, 1981).

The μ-conotoxin family is a group of very basic 22 residue peptides containing three disulfide bonds. The high content of Arg and Lys residues suggests the interaction with a carboxylate group or groups on the Na channel. The effects induced on the Na channel resemble those of an STX blocked channel (Moczydlowski et al., 1986). The selectivity exhibited by these toxins for muscle or electroplax sodium channels suggests that they share a common or overlapping receptor with STX in these tissues not found in neuronal tissues (Ohizumi et al., 1986; Moczydlowski et al., 1986).

Neurotoxins of the second type are lipophilic compounds isolated from several plants, a tropical frog or synthesized in the laboratory. These compounds include BTX, veratridine, aconitine, grayanotoxin, insecticidal pyrethrins, DDT and allethrin (Figure 1.5) (for review see Catterall, 1980; Hille, 1984). Studies with these compounds on several different tissues leads to hyperexcitability and depolarization of the excitable membrane (Schmidt, 1960; Peper and Trautwein, 1967; Deguchi and Sakai, 1967; Ulbricht, 1969; Albuquerque et al., 1971; Herzog et al., 1974). All of these compounds have been found to shift the membrane potential of activation of the Na

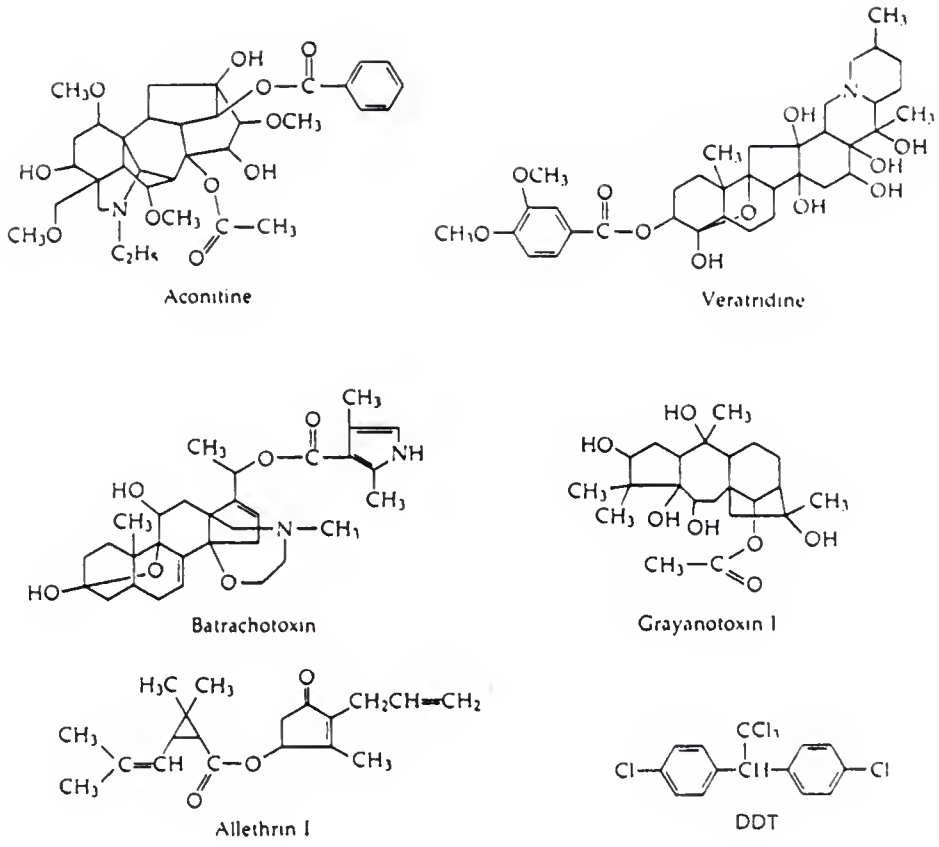

Figure 1:4: Lipophilic alkaloid toxins acting at the site II receptor. Taken from Hille (1984).

channel to more negative values, block inactivation, and reduce ion selectivity. The net result of these effects is the persistent activation of the Na channel. The guanidinium toxins TTX and STX have been shown to block the effects of these compounds noncompetitively (Peper and Trautwein, 1967; Ulbricht, 1969; Albuquerque et al., 1971; Narahashi et al., 1971; Ohata et al., 1973; Schmidt and Schmitt, 1974; Catterall, 1975; Seyama and Narahashi, 1981; Vijvenberg et al., 1982).

Interestingly, the agonist effects of these toxins are all different. As shown by Catterall (1975, 1977a), BTX is a full agonist of the channel in neuroblastoma cells activating approximately 95% of the cells. The other compounds (grayanotoxin, veratridine and aconitine) activated approximately 51%, 8%, and 2%, respectively. Competition displacement experiments have shown that all of these compounds interact competitively with a single class of binding sites in neuroblastoma cells (Catterall, 1977a) and rat brain synaptosomes (Ray et al., 1978). Studies with several different local anesthetics such as dibucaine and diphenhydramine have shown that [^{3}H]-BTX-benzoate, a chemically modified derivative of BTX, is competitively displaced from the site II receptor by these compounds (Creveling et al., 1983). This binding site is believed to reside within the membrane spanning region of the Na channel which would exploit the hydrophobic properties that all of these toxins possess (Catterall, 1980).

An allosteric model for the function of these lipophilic neurotoxins has been proposed by Catterall (1977b). This model utilizes the ideas introduced by Monod, Wyman and Changeux (1965) for allosteric interactions in enzymes. The model is based on the assumption that all of these toxins bind with higher affinity to an activated state of the Na channel and cause a shift in the voltage-dependent equilibrium between the active and inactive states. These toxins bind with high affinity to an active state and translate the energy of binding into shifts in the activation properties of the channel. This causes the channels to remain active (open) at the resting membrane potential (Catterall, 1977b).

The third class of toxins consists of the α-scorpion toxins and the sea anemone neurotoxins. Detailed description of each of these two types of polypeptide toxins will be presented later in the text. Each of these toxin types have been shown to bind to the Na channel and delay the inactivation process (Koppenhofer and Schmidt, 1968; Narahashi et al., 1969). Studies on neuroblastoma cells have shown that both types of toxins stimulate the flux of $^{22}Na^{+}$ in the presence of the alkaloid toxin veratridine (Catterall 1975; Catterall and Beress, 1978; Jacques et al., 1978). Studies <u>in vitro</u> using radiolabeled derivatives of both of types anemone and α-scorpion toxins have shown that anemone toxins displace α-scorpion toxins, but the reverse is not true (Ray et al., 1978; Vincent et al., 1980). Ray et al. (1978) have shown that a positive

heterotrophic cooperativity exists between the site II lipophilic compounds and the α-scorpion toxins. This interaction results in stimulated transport of Na^+, lower K_D values, and an increase in specific binding.

Pharmacological characterization of several neurotoxins isolated from the venoms of *Centruroides suffusus suffusus*, *Centruroides sculpturatus Ewing* and *Tityus serrulatus* resulted in the characterization of a new toxin binding site (Jover et al., 1980; Barhanin et al., 1982; Couraud et al., 1982). These have been classified as the β-scorpion toxins (sequences for several of the β-scorpion toxins are shown in Figure 1:8). These toxins have no effect on the inactivation process like the α-scorpion toxins. The pharmacological effects are manifested in altering the activation of the Na channel. Voltage clamp studies on frog myelinated nerve with *C. sculpturatus Ewing* venom induced repetitive firing due to the appearance of an abnormal Na current upon repolarization of the nerve (Cahalan, 1975). Purified toxins, *C. suffusus suffusus* II (Couraud et al., 1982) and *C. sculpturatus Ewing* IV_a (Wang and Strichartz, 1983), produced similar effects. Kinetic analysis of the Na current after the addition of both α-scorpion and β-scorpion toxins, showed that the same Na channels were modified simultaneously by both toxins (Wang and Strichartz, 1983).

Binding experiments on rat brain synaptosomes with a radiolabeled derivative of TiTXγ, [^{125}I]-TiTxγ, have shown that Css II displaces [^{125}I]-TiTxγ with a $K_{0.5}$ of 900 pM. The K_D

value for [^{125}I]-TiTxτ on this same preparation was determined to be 4 pM. Binding of the β-scorpion toxins has been shown not to be affected by any of the other types of toxins. Depolarization of the membranes, an effect which abolishes α-scorpion toxin binding, has no effect on β-scorpion toxins (Barhanin et al., 1982). Similar results have been obtained using an [^{125}I]-Css II derivative on electroplax membranes (Wheeler et al., 1982); [^{125}I]-TiTxτ has been shown to bind to cardiac Na channel (K_D value of 15 pM) (Lombet and Lazdunski, 1984) as well as to skeletal muscle membranes with a K_D value of 10 pM. However, this same derivative failed to bind to T-tubule membranes (Barhanin et al., 1984). Thus, TiTxτ has the highest affinity for the neuronal, cardiac and surface skeletal forms of the Na channel of any toxin isolated at this time.

The fifth class of neurotoxins which have been pharmacologically characterized includes ciguatoxin and brevetoxin. Ciguatoxin has been isolated from the *Gambierdiscus toxicus*, the dinoflagellate that infects the reef fish *Gymnothorax javanicus* (Chanteau et al., 1976). Structural determination has not been reported for this toxin at this time. Brevetoxins are isolated from the dinoflagellate *Ptychodiscus brevis* (Baden et al., 1979). The structure of brevetoxin B is shown in Figure 1:5. Ciguatoxin (Bidard et al., 1984) and brevetoxin A (Catterall and Gainer, 1985) both bind to the Na channel in neuroblastoma cells and cause repetitive firing as well as stimulation of $^{22}Na^+$ flux in the presence of the

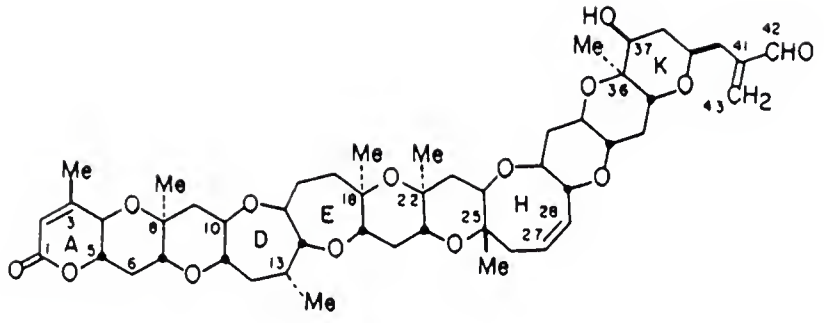

Figure 1:5: Molecular structure of Brevetoxin B. Taken from Nakanishi (1985).

alkaloid site II or the site III sea anemone and α-scorpion polypeptide toxins. The effects of this stimulated transport are blocked noncompetitively by TTX. Brevetoxin causes a three fold increase in the specific binding of [^{3}H]-BTX-B to rat brain synaptosomes at a concentration of 100 ng/ml (Catterall and Gainer, 1985). Ciguatoxin has no effect on the binding of radiolabeled β-scorpion toxin [^{125}I]-TiTxτ, α-scorpion toxin [^{125}I]-AaH II or sea anemone toxin [^{125}I]-As II (Bidard et al., 1984). The synergistic effects associated with the increase in transport have identified this as the newest class of Na channel specific toxins.

Tissue Localization of the Sodium Channel

The voltage-dependent Na channel has been identified in several different types of electrically excitable tissues. The Na channel must have evolved before the separation of the vertebrate and invertebrate species (Hille, 1984). Thus, a physiologically similar Na channel has been observed in mollusks, arthropods, annelids, and vertebrates (Hodgkin and Huxley, 1952; Julian et al., 1962; Frankenhauser, 1963; Goldman and Schauf, 1973; Stampfli and Hille, 1976; Chiu et al., 1979). A major difference between vertebrate and invertebrate nervous tissue is the myelination of many vertebrate axons. Myelination serves as an insulator increasing the cabling properties of the nerve. This, in turn, causes the Na channels to be clustered at the nodes of Ranvier, axonal hillocks and synaptic terminals

(Waxman and Ritchie, 1985). This clustering of Na channels results in the characteristic saltatory nerve impulse seen in myelinated nerves. The density of the Na channels in the nodes has been estimated to exceed 10^3 channels/μm^2 of membrane (Neumcke and Stampfli, 1982). In contrast, the internodal density is less than 25 channels/μm^2 membrane (Ritchie and Rogart, 1977a, 1977b) and synaptic terminals are approximately 25 channels/μm^2 membrane (Ray et al., 1978).

Unmyelinated nerves are considered to have a uniform distribution of Na channels, which causes a continuous mode of impulse conduction. The densities in vertebrate unmyelinated nerves (rabbit cervical vagus nerve) and invertebrate nerves are very similar approximately 110 and 90 channels/μm^2 of membrane, respectively (Ritchie et al., 1976).

Other electrically excitable tissues in which the voltage-dependent Na channel has been characterized include: eel electroplax (Agnew et al., 1978), vertebrate skeletal muscle (Barchi et al., 1979), rat T-tubular membranes (Kraner et al., 1985) and cardiac tissue (Lombet and Lazdunski, 1984). Although the channel densities in these tissues have not been determined directly, binding of radiolabeled guanidinium toxins have provided the following estimates: 560 fmol [^{3}H]-STX/mg protein in cardiac homogenates (Lombet et al., 1981), approximately 10 pmol [^{3}H]-TTX/mg protein in sarcolemma homogenates (Barchi et al., 1979), 228 pmol [^{3}H]-STX/mg protein in eel electric organ homogenates, and 6-10 pmol [^{3}H]-STX/mg protein in T-tubule

membranes (Barchi, 1983). These values are approximately 1/3, 3, 125 and 3 times the [^{3}H]-STX binding in rat brain synaptosomes (Ray et al., 1978). The abundance of Na channels in these membranes has been exploited to yield the purified protein.

Molecular Characterization and Cloning of the Sodium Channel

The Na channel TTX receptor was initially isolated from the electroplax organ of the eel *E. electricus* (Agnew et al., 1978). Improvements in the purification were made by using a monoclonal antibody affinity purification scheme. Using this methodology, (Nakayama et al., 1982) the TTX receptor from electroplax was purified to greater than 90%. Binding of [^{3}H]-STX was observed to decrease through the purification scheme. Agnew and Raftery (1979) found that, following detergent solubilization, addition of phospholipids through the remainder of the purification steps preserved [^{3}H]-STX binding. The purified TTX receptor was observed to be a glycosylated single polypeptide component (M_r = 260,000) with no smaller subunits (Agnew et al., 1978).

More recently, using modern cloning technology Noda et al. (1984) cloned cDNA sequences complementary to the mRNA coding for the Na channel from electroplax. Analysis of the amino acid sequence coded by the cDNA clone has determined the Na channel to consist of 1,820 residues. Sequence analysis revealed four repeated homology domains of approximately 300 residues which are flanked by regions of non-homologous residues. The degree

of homology between the repeats is approximately 50%. This high degree of homology supports the hypothesis that the repeat units all arose from a common ancestral gene which would allow them to adopt a similar secondary structure.

Secondary structure analysis of the Na channel sequence allowed Noda et al. (1984) to propose a model. Each repeat subunit was suggested to consist of six α-helical segments, four of these spanning the membrane while two were proposed to be intracellularly located. The possibility of positioning all six helices in the membrane was also suggested; however, the model they proposed was constructed with only four transmembrane helices per repeat. The four repeats were arranged into a square array where the transmembrane pore is formed by the walls of one of the membrane spanning α-helices per repeat (Figure 1:6). Between repeat subunits II and III, a 200 residue stretch is found which contains four equally spaced clusters of negatively charged residues. Noda et al. (1984) proposed that these negatively charged clusters interact with positively charged residues present in each repeat subunit to form the activation-inactivation gate which is sensitive to a gating charge.

Identification of the Na channel in rat brain was first accomplished using a radiolabeled photoactivatable derivative of scorpion toxin. Covalent attachment of the [^{125}I]-scorpion toxin to the Na channel resulted in the labeling of two components with molecular weights of 250,000 and 32,000

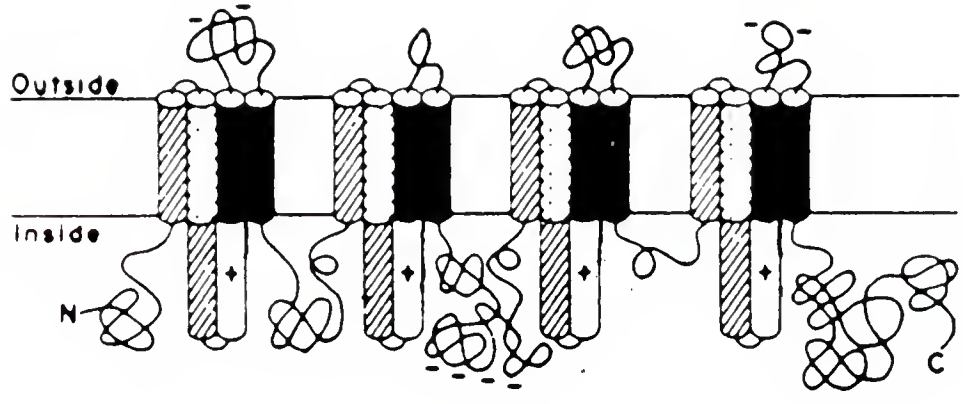

Figure 1:6: Molecular model of the eel electroplax Na channel. Taken from Noda et al. (1984).

(Beneski and Catterall, 1980). Labeling of these components was completely blocked when the rat brain synaptosomes were depolarized or incubated with excess unlabeled scorpion toxin.

The purification of the Na channel from rat brain was similar to that established for electroplax. An absolute requirement for phospholipid during the purification was also observed. The purification resulted in a 1380-fold enrichment of [^{3}H]-STX binding over the starting homogenate. The specific activity was determined to be 2910 pmol [^{3}H]-STX/mg protein or 0.9 mol [^{3}H]-STX per mol of Na channel (Hartshorne and Catterall, 1981, 1984).

The isolated channel was composed of a large 260,000 MW component and two smaller components of 39,000 and 37,000 MW (Hartshorne and Catterall, 1984). These have been classified as the α, β_1 and β_2 subunits, respectively. All three of these subunits are heavily glycosylated. The carbohydrate content by weight constitutes 20% for the α subunit and 36% for each of the two small subunits (Messner and Catterall, 1985). The rat brain Na channel has been proposed to have of a subunit stoichiometry of $\alpha_1(\beta_1)_1(\beta_2)_1$ (Hartshorne and Catterall, 1984). The β_2 subunit has been determined to be disulfide bonded to the large α subunit (Hartshorne et al., 1982), whereas the β_1 subunit is noncovalently associated with the α subunit (Hartshorne and Catterall, 1981). Reconstitution of the solubilized Na channel (Tamkun and Catterall, 1981) and purified channel (Tamkun et al., 1984) into lipid vesicles has shown that this protein does

mediate neurotoxin stimulated $^{22}Na^{+}$ flux; however, α-scorpion toxin binding properties are slightly different. Thus, both rat brain and eel Na channels are composed of a large 260,000 Da component, but the rat brain channel also has two smaller subunits of 39,000 Da and 37,000 Da associated with the large subunit.

Cloning of two different rat brain Na channel mRNAs was recently reported by Noda et al. (1986). Nucleotide sequence analysis of the cDNA clones for these two messages revealed the primary structures for rat brain Na channels I and II. The amino acid sequences of each these clones coded for two large α subunit proteins of 2009 (I) and 2005 (II) amino acids. Furthermore, the sequences of these two show approximately 82% homology to each other and 62% homology to the eel electroplax sequence.

Rat brain Na channels I and II each possess four homologous repeat subunits of 300 amino acid residues. These regions in the rat brain and eel electroplax are highly conserved whereas the cytoplasmic domains show much lower conservation. Secondary structural analysis of the rat brain channel has been used by Noda et al. (1986) to construct a model. The model positions the six α-helical sections (S_1-S_6) of each of the four repeat domains as membrane spanning segments (Figure 1:7). This is contrary to the electroplax model in which they proposed only four transmembrane α-helices per repeat (Noda et al., 1984). The transmembrane pore in the rat brain Na channel is

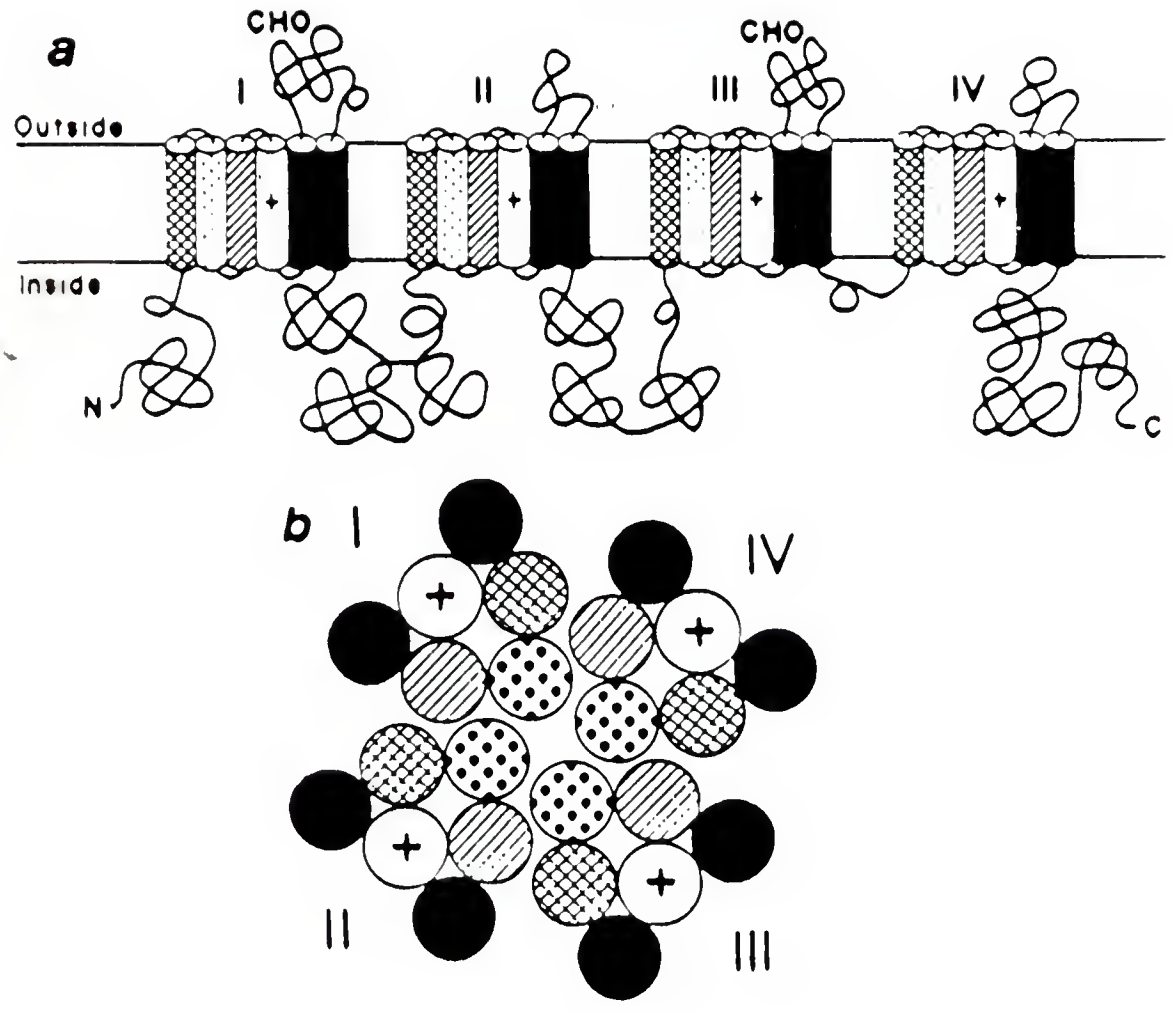

Figure 1:7: Molecular model of rat brain Na channel. Taken from Noda et al. (1986).

suggested to be formed by the walls of the S_2 helix (Figure 1:7, stippled helix) of each of the repeat subunits which are positioned in a square array. Interestingly, the domain of clusters of charged residues observed between repeats II and III of electroplax Na channel are not as prevalent. This newer model of the transmembrane topology of the channel presented by Noda et al. (1986) is based upon theoretical consideration of the measured gating currents which suggest the movement of four to six charged residues across the membrane to open the channel (Hodgkin and Huxley, 1952). This suggests the intramembrane location of many dipoles which only move small distances.

The functional significance of the small subunits present in the rat brain and rat skeletal muscle Na channel is not known. The cloned rat brain mRNA coded for the α subunit of two related Na channels (Noda et al., 1986). Microinjection of cDNA selected mRNA of the rat brain α subunit into *Xenopus laevis* oocytes induced the synthesis of voltage sensitive, TTX sensitive Na channels (Goldin et al., 1986). High salt treatment or incubation at 37 °C selectively removes the $\beta 1$ subunit from purified rat brain Na channels. This has been shown to abolish [^{3}H]-STX binding as well as to eliminate stimulated $^{22}Na^+$ flux in reconstituted vesicles (Messner et al., 1986). More recently, Tejedor et al. (1988) have shown that detergent solubilized rat brain Na channel, following high salt treatment to dissociate the β_1 subunit, reacted with carbodiimides in the absence of added nucleophiles to form

intramolecular isopeptide bonds in the α-subunit. This derivative was shown to have a 3- to 4-fold higher affinity for [^{3}H]-STX. Evidently, the isopeptide bonds are stabilizing the conformation which binds [^{3}H]-STX. This suggests that β_1 is involved in maintenance of the conformation of the Na channel as opposed to directly binding [^{3}H]-STX. Selective removal of the β_2 subunit by mild reduction of the purified Na channel has no effect on either of the binding of [^{3}H]-STX or [^{125}I]-Lqq V or $^{22}Na^+$ flux (Messner et al., 1986). The β_2 subunit is believed to be involved in the intracellular transport and/or membrane insertion of the $\alpha\beta_1$ complex (Schmidt et al., 1985; Schmidt and Catterall, 1986).

Cloning technology has also been exploited to isolate a gene in the fruit fly *Drosophila melanogaster* which codes for a Na channel (Salkoff et al., 1987). The gene codes for a protein which possesses the identical patterning of four repeat subunits of 300 amino acid residues. These repeat subunits contain the same patterning of six α-helical segments that the other cloned channels possess. Homology between the channel sequences was 55% and 51% when compared to rat and eel and sequences, respectively. Sequences that distinguish each of the repeat subunits are absolutely conserved between fly and vertebrate proteins. This supports the theory that a common ancestral gene underwent two rounds of gene duplication to give the modern α subunit structure (Hille, 1984; Salkoff et al., 1987).

Alpha-Scorpion Toxins

The venom delivered by the sting of scorpion is a fairly complex mixture of components including: mucopolysaccharides, phospholipases, hyaluronidase, protease inhibitors, histamine releasers, serotonin, and neurotoxic polypeptides (Couraud and Jover, 1987). Only the Buthidae family of scorpions produce these neurotoxic secretions. This family is further divided into four sub-families by geographical and morphological considerations of which the Buthinae, Centrurinae and Tityinae are the most dangerous (Bucherl, 1971). From these three sub-families, two classes of neurotoxic polypeptides have been identified and classified according to the properties of the Na channel which they modulate. The α-scorpion toxins delay the inactivation, and the β-scorpion toxins affect the activation of the Na channel (Catterall, 1980).

Both classes of these scorpion polypeptides are similar in that they are basic proteins, containing four intramolecular disulfide bonds, with molecular weights of approximately 7,000. In Figure 1:8, the sequences of fifteen different scorpion toxins are shown and classified according to structural homology (Rochat et al., 1979). The positioning of the cysteine residues is absolutely conserved among all of the scorpion toxins sequenced to date. Thus, the positioning of the disulfide bonds is likely to be same in these toxins.

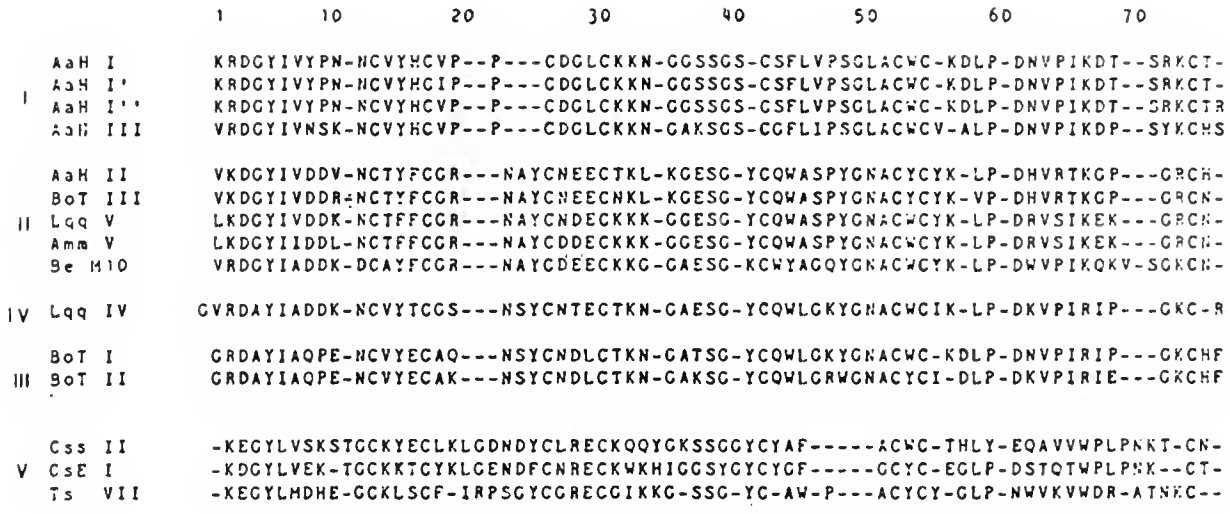

Figure 1:8: Amino acid sequences of scorpion toxins. Taken from Kopeyan et al. (1985).

Although a small number of toxins have been isolated which selectively paralyze insects (Zlotkin et al., 1971; Zlotkin et al., 1979; Lester et al., 1982; Zlotkin et al., 1985) or crustacean species (Zlotkin et al., 1975), the pharmacological effects of the majority of the scorpion toxins appear to be directed towards vertebrate species (Jover and Couraud, 1987). Among the best studied of the vertebrate toxins are the α-toxins AaH II, Lqq V and *Tityus*toxin and β-toxins Css II and TiTxτ. Electrophysiological, pharmacological and structural studies with these toxins have provided much of the data for characterization of the site III and IV receptors on vertebrate Na channels (Catterall, 1980).

Molecular Structure of Scorpion Toxins. The first structure determined by x-ray crystallographic analysis for the α-scorpion toxin variant III from *Centruroides sculpturatus Ewing* at 3.0 Å resolution was reported by Fontecilla-Camps et al., (1980). The structure was refined to a resolution of 1.8 Å in a later report (Almassy et al., 1983). A schematic representation of the α-carbon backbone of this toxin is shown in Figure 1:9. The tertiary structure consists of several loops protruding from a dense core, that contains three of the four disulfide bonds. An α-helical segment of two and one half turns from residues 23-32 and a short three-strand stretch of antiparallel β-sheet are the other prominent structural features of this toxin.

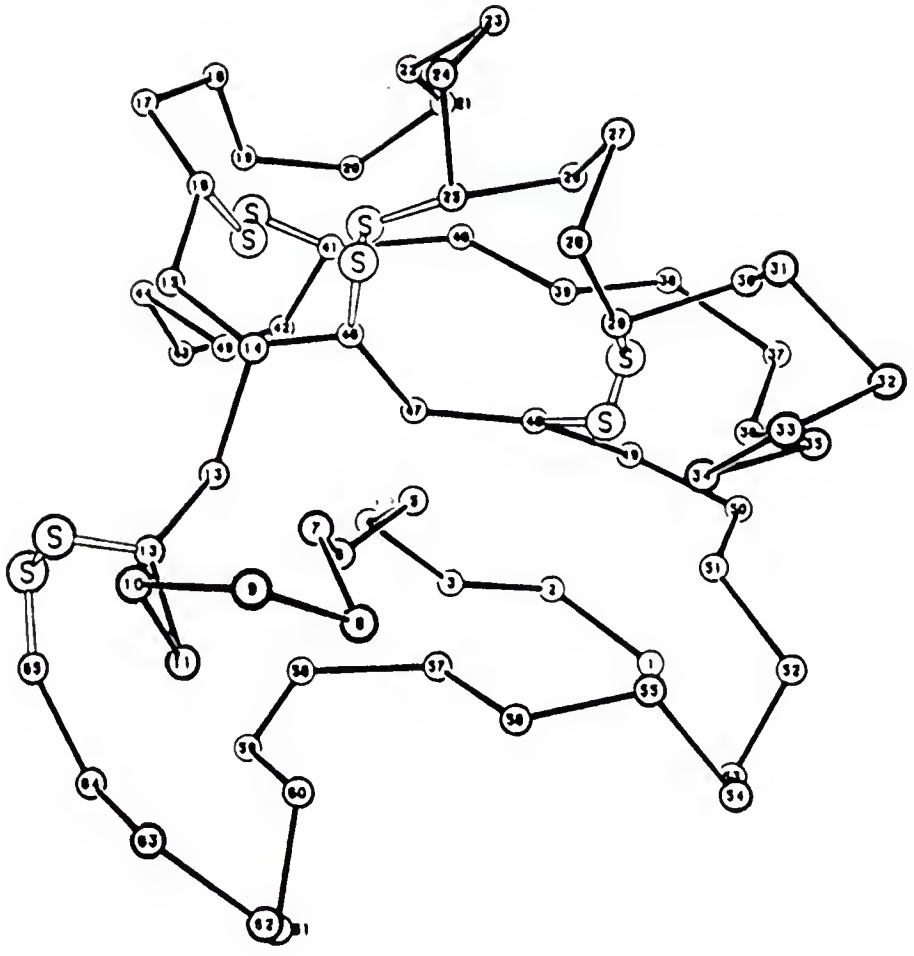

Figure 1:9: X-Ray crystal structure of CsE III. Taken from Fontecilla-Camps et al. (1980).

The molecular structure of this CsE III toxin when compared to the structures of the snake toxins erabutoxin (Low et al., 1976; Kimball et al., 1979), α-cobratoxin (Walkinshaw et al., 1980) and α-bungarotoxin (Agard and Stroud, 1982) show minor similarities. These snake toxins act post-synaptically by binding to the acetylcholine receptor. Both the scorpion and snake toxins are basic proteins containing between 60-75 amino acid residues and either four or five disulfide bonds. They exhibit the same protruding loop-type structure, which originates from a disulfide rich core. However, the snake toxins contain three extended hairpin loops that originate from the core structure, where as the scorpion toxins only have one major loop. Also the α-helical segment and antiparallel β-sheet region are absent from the snake toxins (Almassy et al., 1983).

The molecular structure of CsE III has a relatively high density of aromatic and hydrophobic amino acid residues located on the front surface running from the top to the bottom of the structure (Almassy et al., 1983). This hydrophobic patch contains several conserved amino acid residues present in scorpion toxins. These conserved residues occur at positions 1-4, 47, 52, and 53 in the amino acid sequences of the scorpion toxins. Most of these conserved residues form a hydrophobic patch which nearly spans the width of the molecule (Fontecilla-Camps et al., 1981).

Chemical modification studies on another α-scorpion toxin AaH II suggest that this front hydrophobic surface may be involved

in the interactions by which the biological effects of scorpion toxins are mediated. When a single highly reactive lysine residue is alkylated, AaH II was inactivated (Sampieri and Habersetzer-Rochat, 1978). This lysyl residue is in close proximity to a surface oriented tyrosine residue in the hydrophobic patch of CsE III (Almassy et al., 1983). Reduction and methylation of a single disulfide bridge markedly reduced activity (Habersetzer-Rochat and Sampieri, 1976). Modification of the acidic residues in AaH II resulted in inactivation of the toxin (Sampieri and Habersetzer-Rochat, 1976). Two of the reacted acidic groups in AaH II are located on this front surface patch of CsE III (Almassy et al., 1983). When any of these chemical modifications on AaH II is taken into context with the elucidated structure of CsE III, the front surface region appears to be essential.

Nuclear Magnetic Resonance Structural Properties. Solution structural properties determined by NMR (Krishna et al., 1983) show strong similarities to the crystal structure of CsE III (Fontecilla-Camps et al., 1980; Almassy et al., 1983). At 22 °C, the molecule assumes a very well defined folded structure which undergoes reversible thermal denaturation at 80 °C. Studies on the solution structure of another α-scorpion toxin, *Buthus eupeus* IX, are in agreement with those reported for CsE III (Pastikov et al., 1986). Perhaps the most interesting data obtained by NMR concerns the 35-residue insect toxin, I_5A,

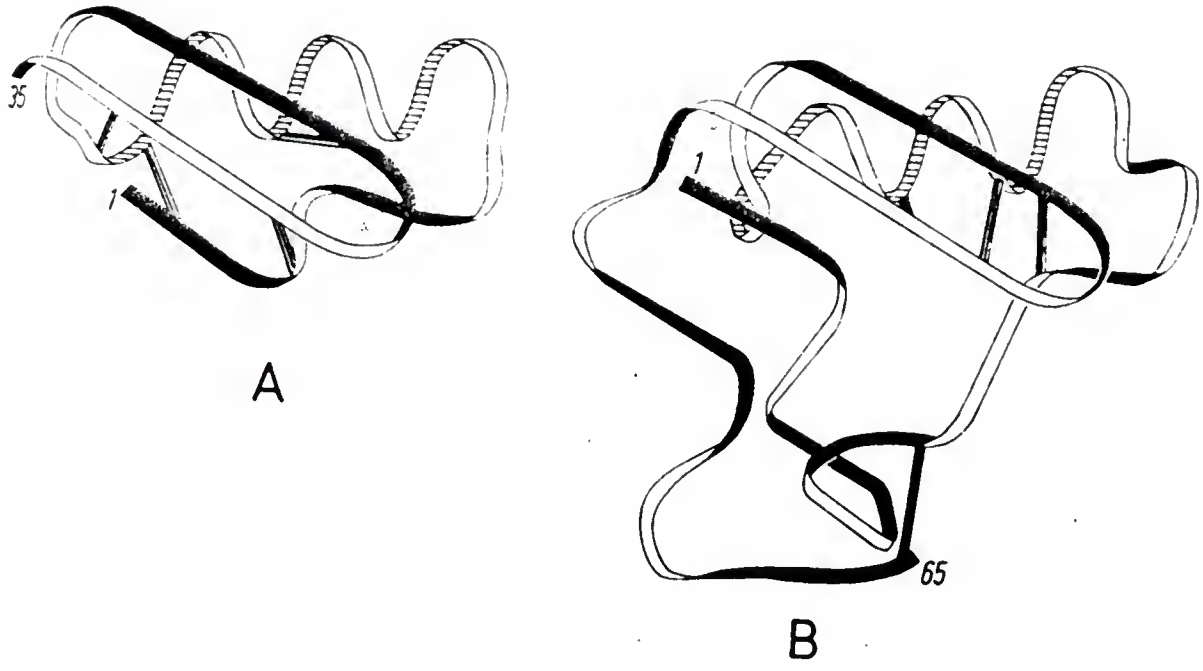

Figure 1:10: Comparison of molecular structure for two different types of scorpion toxins. A, Insect toxin, I_5A, from *Buthus eupeus*. B, Toxin variant III from *Centruroides sculpturatus Ewing*.

isolated from the scorpion *Buthus eupeus* (Arseniev et al., 1984). This toxin is presumed to act post-synaptically on the insect glutamate receptor (Grishin et al., 1982). The primary structure for I_5A shows no similarity to the larger scorpion toxins, but the spatial structures show many similarities (Figure 1:10). The two and one half turn α-helical segment, the three strands of antiparallel β-sheet as well as the same spatial packing of atoms makes these two toxins structurally very similar. These structural elements have been proposed to be responsible for the biological activity of the longer scorpion toxins (Fontecilla-Camps et al., 1982). Therefore, the structural similarities of long and short scorpion toxins are not directly connected with the mechanism of action, and may actually be related to a common ancestral gene (Arseniev et al., 1984).

Immunological Properties of Scorpion Toxins. Scorpion toxins have been classified into five classes based upon structural homology (Rochat et al., 1979). These five classes consist of four α-toxin groups and one β-toxin group. Furthermore, immunocrossreactivity studies have confirmed this structural classification scheme for the four α-toxins (Delori et al., 1981). This data was also corroborated using radioimmunoassay techniques as well (Tessier et al., 1978; El Ayeb et al., 1983a). Despite the structural and antigenic differences, the

α-toxins all bind to the Na channel at the same receptor site (Rochat et al., 1979; Catterall, 1980).

Probing the molecular topology of the AaH II class of α-toxins with antibodies has identified four antigenic domains of the toxin. Initially, El Ayeb et al. (1983b) identified these four domains based upon the binding stoichiometry of four Fab fragments per toxin molecule. Predictions for exposed regions by the method of Hopp and Woods (1981) determined four hydrophilic regions present on AaH II (El Ayeb et al., 1983b). Analysis of thirteen other α-toxins showed four similar hydrophilic regions, suggesting that these domains occur in homologous regions present in the topography of all of the toxins despite variations in the amino acid sequences. These epitopes consist of: (a) residues 23 through 32 which contains the α-helical segment in CsE III (b) residues 30 through 41 (c) residues 50 through 59 which contains a β-turn in CsE III (d) and a discontinuous epitope consisting of residues 5 through 14 disulfide bonded to residues 60 through 64 (El Ayeb et al., 1984).

Peptides representing each of these four antigenic sites have been synthesized and these have been used to prepare monospecific antibodies to each of these determinants (Granier et al., 1984; El Ayeb et al., 1984; Bahraoui et al., 1986). Studies with monospecific antibodies prepared to each one of these sites have shown that each of these epitopes are recognized on native AaH II. However, the monospecific antibody

which recognizes the α-helical segment failed to neutralize toxicity in mice. This same antibody was also determined to retain binding under conditions where AaH II is bound to the Na channel receptor (Bahraoui et al., 1987). Other antibodies directed against the discontinuous epitope containing the disulfide bond or the β-turn region no longer bind to the toxin under the same conditions (El Ayeb et al., 1986). These results suggest that the α-helical segment is not directly involved in the receptor-toxin complex, whereas the β-turn region and the disulfide linked region are involved with the receptor binding site.

Alpha-Scorpion Toxin Receptor. The α-scorpion toxins affect the mammalian neuromuscular system by interacting with the voltage-sensitive Na channel. This interaction results in the delayed inactivation of the Na channel (Koppenhoer and Schmidt, 1968). The result of delayed inactivation is hyperexcitability and a massive release of neurotransmitters (Romey et al., 1976).

Detection of the α-scorpion toxin receptor in a number of tissues was facilitated through the use of [^{125}I]-labeled derivatives of Lqq V or AaH II (Catterall, 1977; Rochat et al., 1977). Binding analyses with these derivatives identified the α-scorpion receptor in neuroblastoma cells (Catterall, 1976; Bernard et al., 1977), rat brain synaptic particles (Ray et al., 1978; Jover et al., 1978), rat skeletal muscle membranes

(Catterall, 1979) and chick cardiac muscle (Couraud et al., 1980).

Identification of the α-scorpion toxin receptor utilized a photoactivatable derivative of [^{125}I]-Lqq V. This derivative upon photolysis was covalently attached to the Na channel of rat brain synaptosomes. Following polyacrylamide gel electrophoresis and autoradiography, two components with molecular weights of 250,000 (α subunit) and 32,000 (β_1 subunit) were identified (Beneski and Catterall, 1980). A later study, using the same photoactivatable [^{125}I]-Lqq V derivative, showed the α-scorpion binding site to be preferentially located on a 34,000 MW β_1 subunit (approximately 85% of the label) and only slight labeling of a large 300,000 MW ($\alpha\beta_2$ complex) component was observed. In this same report, the large subunit upon reduction shifted to a molecular weight 272,000 (α subunit) without affecting the 34,000 MW (β_1 subunit) band. No label was observed to appear on the released 30,000 MW (β_2 subunit) component (Jover et al., 1988). Thus, the α-scorpion receptor appears to be associated with $\alpha\beta_1$ complex possibly near the interface of these two noncovalently associated subunits.

Solubilization of the rat brain Na channel destroys the binding of the α-scorpion toxins (Catterall et al., 1979). When the solubilized Na channel is reconstituted into phospholipid vesicles, binding of α-scorpion toxins is recovered. The reconstituted Na channel possesses the three major toxin binding sites (Tamkun and Catterall, 1981a, 1981b), and it facilitates

Na^+ transport in the presence of veratridine (Talvenheimo et al., 1982).

With the successes of purification and reconstitution of the Na channel, conditions were established which either the β_1 or β_2 subunits were selectively removed from the complex. These experiments determined that removal of the β_2 subunit, leaving the $\alpha\beta_1$ complex, did not affect α-scorpion toxin binding, [^{3}H]-STX binding or Na^+ flux in the presence of veratridine. However, selective removal of the β_1 subunit abolished α-scorpion toxin binding as well as [^{3}H]-STX binding. This $\alpha\beta_2$ complex was unable to mediate veratridine stimulated Na^+ flux (Messner et al., 1986). Thus, $\alpha\beta_1$ complex appears to display all of the functional characteristics of the Na channel.

The binding of α-scorpion toxins to the Na channel receptor in rat brain synaptosomes (Ray et al., 1978; Jover et al., 1978) and neuroblastoma cells (Catterall, 1976; Bernard et al., 1977) have shown K_D values of approximately 1 nM. The binding of the α-scorpion toxins are greatly affected by the membrane potential. Upon depolarization of neuroblastoma cells (Catterall, 1977) and synaptic particles (Ray et al., 1978), the $K_{0.5}$ values increased significantly and the binding of [^{125}I]-Lqq V was inhibited. The α-scorpion toxin binding site showed no competition with any other toxins acting on the Na channel, except for the type 1 sea anemone toxins (Ray et al., 1978; Jover et al., 1980; Schweitz et al., 1985).

A final characteristic of the α-scorpion toxin receptor is the synergistic interaction this site experiences with the site II lipophilic alkaloid toxins such as BTX and veratridine (Catterall, 1977a; Ray et al., 1978). These two binding sites exhibit positive heterotrophic cooperativity which results in markedly increased binding of [^{125}I]-Lqq V (4-fold enhancement in the presence of BTX) (Ray et al., 1978). An allosteric model has been presented by Catterall (1977b) to describe this behavior. This model assumes two distinct states for the Na channel: active and inactive. The alkaloid toxins bind preferentially to the active state, scorpion toxins enhance activation by alkaloid toxins by lowering the equilibrium constant for the transition between the two states.

Angelides and Brown (1984) have mapped the distance between the alkaloid site and the α-scorpion site using a fluorescent derivative of BTX. In these studies, the effect of binding Lqq V caused a 20 nm red shift in the fluorescence emission maxima of the BTX derivative, indicating the BTX derivative was experiencing a more hydrophilic environment. Fluorescence resonance energy transfer measurements showed that the distance separating the two receptors was approximately 37 Å. Also, using the same measurement techniques, the TTX receptor was 35 Å from the α-scorpion receptor. Upon binding of BTX, this distance increased to 42 Å indicating a conformational change associated with the binding of BTX (Angelides and Nutter, 1984).

Sea Anemone Neurotoxins

The phylum coelenterata is composed of animals which exhibit one of the simplest levels of organization in the Metazoa. All members of this phylum possess stinging nematocysts or cnidae which are used to paralyze prey to facilitate its capture. The nematocyst consists of a coiled hollow thread, contained within a chemically or mechanically triggered capsule. Upon stimulation, the nematocyst discharges, and the thread uncoils delivering toxins through the hollow thread (Rathmayer, 1979).

Richet (1903) was the first to investigate the toxic components of the sea anemone *Anemonia sulcata*. Beress et al. (1975) succeeded in isolating two polypeptides from this anemone. Purification of toxins from several other anemones followed the successful establishment of a purification scheme. The primary structures for several of these toxins were determined in a number of different labs (Fig. 1.11). As shown in Figure 1:11, the sequences of eleven toxins are aligned such that the positioning of the six cysteine residues is conserved. These cysteine residues form three intramolecular disulfide bonds in all of these toxins. Only the disulfide pairs of As II have been chemically determined. These are between Cys_4 and Cys_{46}, Cys_6 and Cys_{36}, and Cys_{29} and Cys_{47} (Wunderer, 1978). It is believed that the same disulfide pairs are present in the other toxins due to the conservation of the cysteine residue positions in the sequences. Other conserved residues include positions 6 and 8, Gly_9, Pro_{10}, Arg_{13}, Gly_{19}, Trp_{30}, and Lys_{47}.

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          1     5     10    15    20        25    30    35    40    45
Af I    G V A C L C D S D G P N V R G N T L S G T I W L   A G C P S G W H N C K A H G P T I G W C C K Q
Af II G G V P C L C D S D G P S V R G N T L S G I I W L   A G C P S G W H N C K A H G P T I G W C C K Q
As I    G A P C L C K S D G P N T R G N S M S G T I W V   F G C P S G W N N C E G R A  I I G Y C C K Q
As II   G V P C L C D S D G P S V R G N T L S G I I W L   A G C P S G W H N C K K H G P T I G W C C K Q
As V    G V P C L C D S D G P S V R G N T L S G I L W L   A G C P S G W H N C K K H K P T I G W C C K
Ax I    G V S C L C D S D G P S V R G N T L S G T L W L Y P S G C P S G W H N C K A H G P T I G W C C K Q
Ax II   G V P C L C D S D G P R P R G N T L S G I L W F Y P S G C P S G W H N C K A H G P N I G W C C K K

          1     5     10    15    20        25    30    35    40    45
Hm III  G N C K C D D E G P Y V R T A P L T G Y V D L   G Y C N E G W E K C A S Y Y S P I A E C C R K K K
Hp II   A S C K C D D D G P D V R S A T F T G T V D F   W N C N E G W E K C T A V Y T P V A S C C R K K K
Hp III  G N C K C D D E G P N V R T A P L T G Y V D L   G Y C N E G W E K C A S Y Y S P I A E C C R K K K
Sh I    A A C K C D D E G P D I R T A P L T G T V D L   G S C N A G W E K C A S Y Y T I I A D C C R K K K
```

Figure 1:11. Amino acid sequences of sea anemone long polypetide toxins affecting sodium channels.

Also shown in Figure 1:11, is the classification of these sequences into two distinct classes based upon sequence homology. Among the members of each group, there exists approximately 60% homology, and between the two groups there exists approximately 30% homology. Immunochemical studies support this classification system (Schweitz et al., 1985; Chapter 2, this dissertation). Furthermore, the members of the type 1 class all belong to the Actiniidae family and the type 2 class all belong to the Stichodactylidae family (Pennington et al., 1987).

A third class of smaller sea anemone toxins containing 31 amino acids have been isolated from two different anemones. Toxin III from *Anemonia sulcata* (Martinez et al., 1977) and PaTX from *Parasicyonis actinostoloides* (Nishida et al., 1985) are the only two examples of these small anemone toxins. Interestingly, they are active only on crustaceans (Martinez et al., 1977; Fujita and Warshina, 1980). Furthermore, these small anemone toxins have four disulfide bonds as opposed to three in the longer toxins.

Molecular Structures of Long Sea Anemone Toxins. Although the crystal structure for an anemone toxin has not been reported, a considerable amount of information about the solution structures of several anemone toxins has been reported. Initially, Prescott et al. (1976) used laser Raman spectroscopy to investigate the structural properties of As II. They

reported that the characteristic absorption peaks for α-helices and β-structure were not observed, and concluded that the peptide backbone is predominantly disordered. Application of laser Raman spectroscopy, CD, fluorescence emission, NMR rotational correlation time, and Chou-Fasman rules on Ax I suggested that this toxin was roughly spherical with numerous β-turns, some β-sheet structure and possibly some α-helix structure. The aromatic residues Trp_{23} and Tyr_{25} appear to be exposed to the solvent, whereas one or more of the other Trp residues appear to be buried (Ishizaki et al., 1979). Norton and Norton (1979) using ^{13}C-NMR, determined that Tyr_{25} of Ax I is exposed to the aqueous environment. Furthermore, many resonances that were assigned were very similar to those of model small peptides suggesting that Ax I lacks extensive structure. One particularly low pK_a of 2.0 was determined for one of the Asp side-chain carboxylates. Normal pK_a values for this carboxylate in small peptides is approximately 3.9 (Keim et al., 1973).

Natural abundance ^{13}C-NMR studies on As II determined that the conformation of As II is very similar to Ax I. Approximately half of the aromatic resonances and nearly all of the carboxylate resonances were identical between the two toxins. A similar low pK_a for an Asp carboxylate sidechain was observed (Norton et al., 1980). This low pK_a is proposed to result from the formation of a salt bridge with the ∈-amino group of Lys_{37}

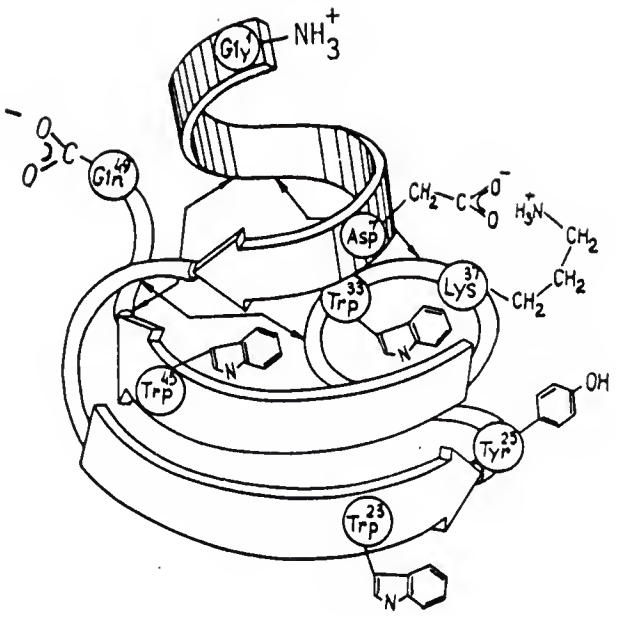


Figure 1:12: Proposed model of the molecular structure of Ax I. Taken from Nabiullin et al. (1982).

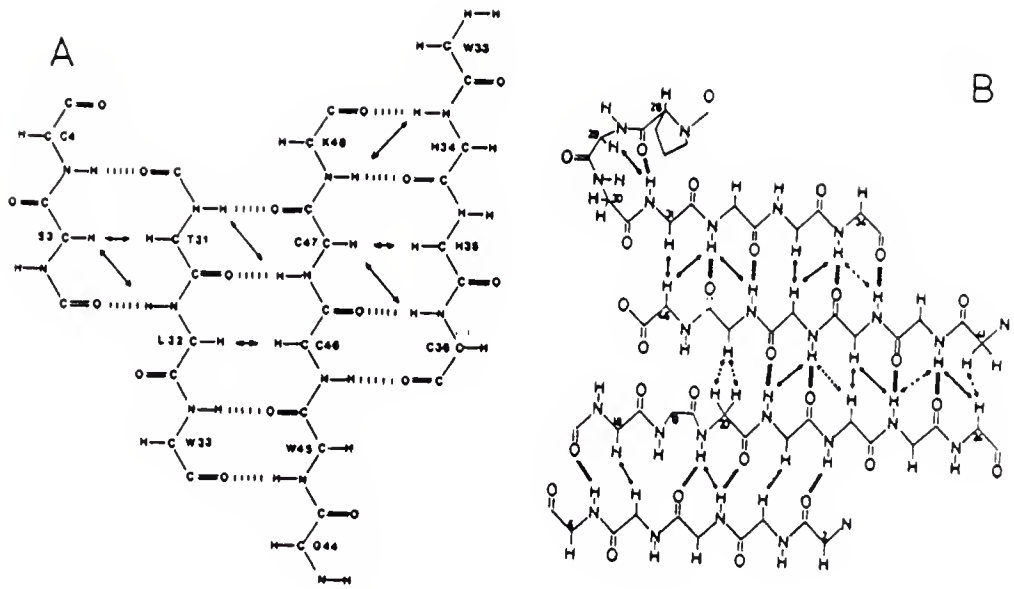


Figure 1:13: Antiparallel β-sheet structures in Type 1 Sea Anemone Toxins. A, As II (Gooley et al., 1986). B, As I_a (Widmer et al., 1987).

(Norton and Norton, 1979; Norton et al., 1980). Using ^1H NMR to study the structure of As I, the same general structural properties as those of As II and Ax I were observed. The structure appears to be generally open without a hydrophobic core. An abnormally low pK_a for an Asp carboxylate sidechain was observed. Interestingly, the As I sequence has a Lys substitution for the Asp_7 present in the As II and Ax I sequences. Thus, the salt bridge most likely involves the Asp_9 in all three of the toxins (Gooley et al., 1984a).

A preliminary model of Ax I was reported by Nabiullin et al. (1982). This model (shown in Figure 1:12) consists of a short N-terminal α-helix, a two strand antiparallel β-sheet and several β-turns. More recently, 2D-NMR techniques have been applied to both Ax I and As I (Norton and Gooley, 1986; Widmer et al., 1988). In both cases, the major secondary structural element identified was four strands of antiparallel β-sheet as shown in Figure 1:13. Also identified was a type II reverse turn between residues 30 through 33 in Ax I (Gooley and Norton, 1986) and between residues 28 through 31 in As I_a (Widmer et al., 1988). (The previously reported sequence of As I contained an ambiguity at position 3. Further purification resolved the ambiguity to the presence of two isotoxins which contained only a single amino acid sequence difference at position 3). No α-helical region was detected in either of Ax I or As I_a. Aromatic resonances have identified a hydrophobic cluster on the surface of Ax I, As I and As II. The aromatic residues

determined to be involved in this are Trp_{23} and Tyr_{25} (Gooley et al., 1986). Conformational heterogeneity in the As II and Ax I molecules has also been observed. The conformational change has been proposed to occur due to the *cis-trans* isomerization of the Gly_{40}-Pro_{41} peptide bond. The As I molecule was not observed to display this behavior (Gooley et al. 1984b). These NMR studies have revised the original model presented by Nabiullin et al. (1982), in that no α-helical region is formed, the β-sheet structure is much more extensive, and the positioning of the β-turns are slightly different.

These 2D-NMR techniques have recently been applied to type II anemone toxins. Wemmer et al. (1986), using 2D-NMR, revised the sequence originally reported by Schweitz et al. (1985) for Hp II. In this report, they identified two sequences of β-sheet most likely joined in a discontinuous distorted sheet, connected by β-turns and extended loops without any α-helical regions. The secondary structural elements of ShN I have been reported by Fogh et al. (in press) The structural elements are much the same as for Hp II, a large four strand antiparallel β-sheet, β-turns and no α-helical structure. The Trp_{30} residue appears to be oriented to the aqueous environment and at least one of the Tyr residues of ShN I is accessible to flavin dye (Norton et al., in press).

Immunochemical Studies. As previously mentioned, the differentiation of long sea anemone toxins into at least two

classes is based upon structural as well as immunochemical methods. Schweitz et al. (1985) were able to develope an anti-Hp II antibody which showed no reactivity with any type 1 anemone toxin tested. We have also developed a radioimmunoassay with an anti-ShN I antibody, which we also determined it to have no immunocrossreactivity with any of the type 1 toxins tested (Chapter II, this dissertation).

Immunochemistry has also allowed the surface structure of the type 1 toxins to be probed. El Ayeb et al. (1986) isolated monoregion-specific antibodies to As I and As II based upon the stoichiometry of Fab binding to As II. The region recognized was determined to contain Asp_7, Asp_9 and Gln_{47} of As II. Furthermore, this site remained accessible to the antibody when As II was bound to the rat brain synaptosomal Na channel receptor. These studies suggest that this region is not directly involved in the receptor toxin complex.

Chemical Modification Studies. Chemical modification experiments to determine which residues may be involved in binding to the receptor surface have yielded some information; however, many conflicting reports have caused confusion in identifying these residues. The first modifications reported were the incorporation of radioactive iodine, [^{125}I], into histidine residues of As I and As II (Hucho, 1978; Habermann and Beress, 1979). In both cases, radiolabeling the polypeptide did not result in a significant loss of activity. The [^{125}I]-As II

derivative was used to characterize the receptor in rat brain synaptosomes (Vincent et al., 1980). Determination of the site of iodination was later reported to be His_{37} (Barhanin et al., 1981). This derivative was determined to possess greater than 80% of the toxicity of the native toxin. Carbethoxylation modification of histidine residues decreased the toxicity of As II on crabs and mice by factors of 5 and 13, respectively. Reversal of this modification resulted in complete recovery of the native toxin toxicity (Barhanin et al., 1981).

The role the conserved Arg_{14} residue has been investigated using reagents which specifically modify the guanidinium group. Vincent et al. (1980) and Barhanin et al. (1981) determined that modification with 1,2 cyclohexanedione abolished the binding and toxic properties of As II. Other groups have presented conflicting data where Arg modification did not destroy the activity (Kolkenbrock et al., 1983). Newcomb et al. (1980) using phenylglyoxal reported that Arg modification did not abolish activity of Ax I. The absolute conservation of this residue among all anemone toxins implies its importance in either structural maintenance or in the binding domain.

The role of amino groups has been probed with reagents which retain a charge or eliminate the charge associated with either the α-amino group or the $\in$-amino group of lysine residues. The results indicated that conservation of charge by guanidylation of the Lys residues did not affect activity. However, acetylation or treatment with fluorescamine resulted in

derivatives with activities which were reduced by factors of 8 and 14, respectively, on crabs and mice (Baharnin et al., 1981). Specific modification of the α-amino group or the ∈-amino group of Lys_{35} of As II, through reduction of the Schiff's base that forms when reacted with pyridoxal phosphate, was used to introduce a negative charge at these residues. This modification reduced the activity by a factor of three (Stengelin et al., 1981). Thus, the basic groups of As II are important in preserving the toxic properties of the molecule.

Chemical modification of the carboxyl groups of As II by carbodiimide-mediated amidation yielded derivatives which were modified at all three, at two or at one of the carboxyl groups. Only the derivative that was modified at all three carboxyl groups was studied in detail (Barhanin et al., 1981). They reported that this derivative was devoid of the biological properties of the native toxin, however, this derivative bound to rat brain synaptosomes with a similar affinity to that of the native toxin. They describe this derivative as a competitive antagonist of the native toxin. Although the mono- and di-substituted derivatives failed to kill crabs or mice at concentrations up to 10 times the LD_{50} of the native toxin, both of these derivatives were reported to bind to rat brain synaptosomes with affinities identical to the native toxin. Gruen and Norton (1985) reported that carboxyl modification on Ax I resulted in modification of both Asp_7 and Asp_9. The

conformation of this derivative was greatly altered and the biological activity was lost.

Chemical modification of only one type 2 toxin has been reported (Kozlovskaya et al., 1982). They reported that modification of arginine residues abolished the activity of Hm I. Modification of the indole ring of the tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide reduced the activity of Hm I by a factor of 2. Modification of the lysine residues with 2,4 pentanedione resulted in a derivative with only 10% activity of the native toxin. Thus, the basic amino acid residues appear to be essential for activity and the tryptophan residue does not appear to be as essential.

Pharmacological Properties. Investigation of the pharmacological properties of anemone toxins were initiated with the purification of a neurotoxic protein (CTX) from *Condylactis gigantea* and the subsequent electrophysiological characterization. Narahashi et al. (1969) applied CTX to squid giant axon and observed the prolongation of the action potential due to the delayed inactivation of Na channel. These properties resembled those of α-scorpion toxins applied to the same preparation (Koppenhofer and Schmidt, 1968). With the success of isolating pure neurotoxins from several other anemones, determination of the toxic properties on invertebrates and vertebrates was studied. Beress et al. (1975) observed a wide range of toxicities for the isotoxins isolated from *Anemonia*

sulcata. The LD_{50} values for the toxins isolated from several different anemones are shown in Table 1:1. One of the more interesting aspects of these toxins is the wide range of toxicities that these toxins possess for crabs and mice, especially when two toxins in the same class, (Hm III and ShN I), represent the two extremes. These two toxins have nearly 80% sequence identity (Fig 1:11), yet the toxicities that they display on crabs and mice are inversely related. Assuming that the species selectivity of these two toxins results from the differences in the amino acid sequence differences between the two toxin molecules, it may be possible to determine which of these residues confers mammalian versus crustacean activity.

The binding properties of anemone toxins were first investigated in experiments where [^{125}I]-Lqq V was competitively displaced by As II from neuroblastoma cells with an apparent K_D of 90 nM (Catterall and Beress, 1978). Furthermore, they showed that the binding of As II decreased upon depolarization of the cells and that As II stimulated the flux of $^{22}Na^+$ in the presence of veratridine. Investigation of the binding to rat brain synaptosomes showed that As II displaced [^{125}I]-Lqq V with a K_D of 400 nM (Ray et al., 1978). Use of the radiolabeled [^{125}I]-As II derivative on rat brain synaptosomes determined that unlabeled As II displaces the labeled As II with a K_D of 240 nM (Vincent et al., 1980). In this same report, they showed that As II competitively displaces [^{125}I]-AaH II with a K_D of

Table 1:1. Pharmacological Properties of Purified Sea Anemone Toxins

Toxin	Toxicity (μg/kg) Crab LD_{50}[a]	Toxicity (μg/kg) Mice LD_{50}[b]	Rat Brain Synaptosome K_D (nM)
ShN I	3	>15,000	31,000
As I	8	4,000	7,000
As II	8	100	150
As III	14	>18,000	>10,000
As V	20	19	50
Hp III	26	53	300
Sg I	28	>2,000	>10,000
Hp II	40	4,200	>100,000
Ax I (*Anthopl.* A)	44	66	120
Ax II (*Anthopl.* B)	160	8	35
Hp IV	230	40	10,000
Hm III	820	2	---

[a]Injection into intrahemocoelic space.

[b]Intraperitoneal injection.

(Taken from Kem, 1988).

200 nM but that AaH II does not displace [^{125}I]-As II. Calculation of the As II receptor density was reported to be 10 times that of the AaH II receptor. The authors suggest the possibility of different Na channel classes of which AaH II recognizes only a single type.

The identification of a second class of anemone toxins has recently been reported (Schweitz et al., 1985; Zykova et al., 1986; Metrione et al., 1987; Kem et al., submitted). Binding properties for rat brain synaptosomes showed that Hp toxins were unable to displace [^{125}I]-As V. All Hp toxins except Hp II were able to competitively displace [^{125}I]-AaH II. The Hp toxins did not affect the binding of TiTxγ (Schweitz et al., 1985). It is our goal to further characterize the binding properties of this class of anemone toxins.

Cardiac Properties. As described earlier in this chapter, cardiac tissue possesses a Na channel which exhibits similar toxin binding properties as neuronal Na channels. Interestingly, cardiac tissue appears to contain Na channel which is relatively insensitive to STX (Catterall and Coppersmith, 1981a, 1981b). Cultured cardiac cells displaying this same property towards STX have different properties for anemone toxins and α-scorpion toxins. In neuronal tissue the K_D for Lqq V and As II are 2 and 200 nM, respectively, whereas in cultured cardiac cells, the $K_{0.5}$ values are 120 and 20 nM, respectively, (Catterall and Coppersmith, 1981a, 1981b). As II

was also observed to exhibit biphasic behavior compared to the hyperbolic results obtained with Lqq V.

Studies where As II has been applied to guinea pig atria show that it causes long prolongation of the action potential, and that it acts as a positive inotroph (Ravens, 1979). Ax I, another type 1 anemone toxin, has been shown to possess very strong positive inotroph properties (Shibata et al., 1976, 1978). Ax II, an isotoxin of Ax I, has been determined to have a 12.5-fold higher cardiac stimulant activity than Ax I or As II (Reiner et al., 1985). Type 2 anemone toxins (Hp) showed only a moderate increase in contractility relative to that of As II, Ax I or Ax II (Renaud et al., 1986). Thus, the type 1 anemone toxins appear to possess much stronger cardiac binding properties than the type 2 anemone toxins. This is consistent with the existence of at least two separate anemone toxin receptors.

Ax I has been determined to have a 200 fold greater inotrophic activity than digoxin (on a molar basis) and a higher therapeutic index in vivo (Scriabine et al., 1979). The mechanism of action of Ax I has been determined to involve a delay in the inactivation of the cardiac Na channel, causing a prolongation of the Na^+ current (Kodama et al., 1981) without affecting neuromuscular function (Scriabine et al., 1979). Thus, the potential exists to use these type 1 anemone toxins as templates from which drugs designed for the treatment of

congestive heart failure may be fashioned (Shibata and Norton, 1982).

Unanswered Questions

Although the sea anemone toxins have been utilized in the intense study of the Na channel in recent years, there are still a number of unanswered questions relating to toxin structure, the species selectivity, receptor characterization and binding properties. In this dissertation, the binding properties of ShN I to the Na channel receptor in crustacean and mammalian *in vitro* systems are investigated through use of a radiolabeled derivative of ShN I. A program was initiated to chemically synthesize, refold, and purify the native 48-residue polypeptide. Furthermore, elucidation of several residues which are essential for toxicity as well as receptor binding were determined through the preparation of six monosubstituted synthetic analogs directed at the N-terminal domain of ShN I. Chemical and physical methods have been used to study the refolded synthetic products. The information obtained from these studies is expected to give the first detailed characterization of the type 2 anemone toxin receptor of the Na channel.

CHAPTER II

CHARACTERIZATION OF A NEW SEA ANEMONE NEUROTOXIN RECEPTOR SITE ASSOCIATED WITH THE SODIUM CHANNEL IN RAT BRAIN SYNAPTOSOMES AND BLUE CRAB AXOLEMMA VESICLES

Introduction

The Na channel is the transmembrane, glycoprotein responsible for the selective passage of Na^+ ions through the lipid bilayer in electrically excitable tissue. A variety of pharmacological agents have been utilized to investigate the mechanism of this ion channel. The number of different binding sites continues to increase, but at this time at least five different classes have been identified (for review see Catterall, 1986). These five classes are as follows: (i) tetrodotoxin (TTX) and saxitoxin (STX) block the entry of Na^+ ions through the channel by preventing the opening of the channel (Narahashi, 1974; Ritchie and Rogart, 1977; Ritchie, 1980); (ii) the lipophilic alkaloids [e.g., batrachotoxin (BTX) and veratridine as well as pyrethrins bind to an open form of the channel and stabilize it in this conformation (Ulbricht, 1969; Albuquerque and Daly, 1976; Narahashi, 1976; Jacques et al., 1980); (iii) the α-scorpion and sea anemone polypeptide toxins delay the inactivation of the channel (Romey et al., 1975, 1976; Bergman et al. 1976), and have also been shown to have positive heterotropic cooperativity with the site II lipophilic toxins

(Ray et al. 1978; Catterall and Tamkun, 1981); (iv) the β-scorpion toxins such as those isolated from *Tityus serratulus* alter the activation properties of the channel (Vijvenberg et al., 1984; Barhanin et al., 1984); (v) ciguatoxin and brevetoxin bind to a specific class of sodium channels (Bidard et al., 1984; Huang et al., 1984).

Recently, a novel class of anemone toxins have been isolated from the *Stichodactylid* sea anemone family (Schweitz et al., 1985; Zykova et al., 1986; Kem et al. 1986). These toxins differ significantly from the *Actiniid* toxins in amino acid sequence homology. Members of each class of anemone toxins possess greater than 60% sequence identity. However, only about 30% identity exists between the two classes. The major contribution to the interclass sequence identity is the positioning of cysteine residues. As a result of the cysteine residue positioning, it is believed that all anemone toxins possess the same disulfide pairings although *Anemonia sulcata* II (As II) is the only toxin for which the disulfide pairs have been reported (Wunderer, 1978).

Probing the two classes of sea anemone toxins for immunocrossreactivity has shown that there is no cross reactivity between polyclonal antibodies against *Heteractis paumotensis* (formerly *Radianthus paumotensis*) III, (Hp III) and As II, As V, Ax I, or Ax II (Schweitz et al. 1985).

In binding studies using rat brain synaptosomes, Hp II was unable to displace [^{125}I]-As V, [^{125}I]-AaH II (*Androctonus*

austrialis Hector II) or [^{125}I]-TiTx$_\gamma$. However, Hp III and other *Heteractis paumotensis* toxins were successful in displacing [^{125}I]-AaH II but not the [^{125}I]-As V or [^{125}I]-TiTx$_\gamma$ (Schweitz et al., 1985).

Our group recently isolated and sequenced another *Stichodactylid* toxin from the sea anemone Stichodactyla helianthus (Kem et al., submitted). The pharmacological properties of this toxin are among the most interesting of any sea anemone toxin isolated to date. This toxin has the greatest range of selective toxicity reported for sea anemone toxins. The LD_{50} for crustaceans is 5000 fold lower than the LD_{50} for mammals (Kem et al., submitted).

In this report, we examine the electrophysiological effects of ShN I on lobster olfactory somas. Secondly, we describe the preparation of a mono-iodinated derivative of Stichodactyla helianthus neurotoxin I ([^{125}I]-ShN I). The [^{125}I]-ShN I derivative was then used to characterize the pharmacological properties of ShN I on rat brain synaptosomes and Blue Crab walking leg axolemma vesicles. Both of these preparations have been well characterized, and have been shown to be enriched in the sodium channel protein. Use of these two systems may help to identify the existing differences between them. Finally, using a polyclonal antibody prepared against ShN I, we have examined the immunocrossreactivity between type 1 and type 2 anemone toxins.

Experimental Procedures

Materials. Sea anemone toxin I from *Stichodactyla helianthus* (ShN I) was purified as described previously (Kem et al., submitted). [^{3}H]-STX (specific activity 10 Ci/mmol) was kindly provided by Dr. Gary Strichartz (Department of Anesthesia, Harvard Medical School, Boston Massachusetts). *Androctonus austrialis Hector* II was a kind gift from Dr. Herve Rochat (Laboratoire de Biochimie, Marseille, France). *Anemonia sulcata* II and *Bolecera tuediae* II were kindly provided by Dr. Lazlo Beress (Institut fur Meereskunde an der Universitat Kiel, Kiel, West Germany). Na^{125}I was purchased from New England Nuclear (Boston, Massachusetts) and had a specific activity of 2200 Ci/mmol. Di-O-C_5 (3,3'dipentyloxacarbocyanine iodide) was obtained from Molecular Probes, Inc. (Junction City, Oregon). Staphylococcus Protein A was purchased from Boehringer Mannheim (Indianapolis, Indiana). Affi-gel 15 was purchased from Bio-Rad Inc., (Richmond, California). Antisera to ShN I was obtained from New Zealand White Rabbits. TTX was obtained from Calbiochem (La Jolla, California). Trypsin, collagenase (Type V) and veratridine were obtained from Sigma Chemical Co., (St. Louis, Missouri). All other reagents were the highest commercial grade available.

Biological Assay. Intrahemocoelic injection of 3-5 g fiddler crabs (*Uca pugilator*) was performed with purified ShN I diluted at a constant interval with normal saline containing 0.1 mg/ml

BSA (142 mM NaCl, 2 mM $CaCl_2$, 40 mM KCl, 9 mM Dextrose, and 10 mM Tris HCl, pH 7.4). Paralytic response was determined 15 min following the injection by placing the animals on their backs and measuring their ability to "right" themselves in a 2 min interval. Intracerebroventricular injection of 24-31 g white mice was performed with a constant dose interval of ShN I. At low doses (1-2 μmol/kg), a slight tremor could be detected by holding the animal upside down by its tail. At higher doses, the injection resulted in paralysis and eventual death of the animal.

Preparation of lobster olfactory somas. Antennules excised from the Florida spiny lobster, *Panulirus argus*, were perfused with cold saline to remove the hemolymph, and cut into 1 cm sections which were split longitudinally. These sections were bisected again longitudinally. The half that bore the axon bundles was discarded and the half that bore the sensillia contained the receptor cells. The clumps of receptor cells were dislodged from the connective tissue with a gentle stream of saline from a pipette. Isolated receptor cells were obtained from these clumps, by dissociating them with the enzymes collagenase followed by trypsin. Cells were treated with 100 IU/ml of collagenase in saline for 90 min, with gentle agitation, followed by 30 min in 0.4-0.6 mg/ml trypsin in Ca^{2+}-free saline. The cells were then rinsed several times in normal saline and transferred to the recording bath. All recordings

were done in normal saline (140 mM NaCl, 2mM $CaCl_2$, 5.4 mM KCl, 9mM dextrose and 10 mM Hepes, pH 7.4)

Intracellular lobster olfactory soma recording. Recordings on the isolated receptor cells was according to the method of Anderson and Ache (1985). Briefly, preparations were examined at 200 X using a fixed stage microscope (Aus Jena) equipped with Modulation Contrast optics. Intracellular recordings were obtained with the whole-cell, patch clamp technique (Hamill et al., 1981). High impedance (>1 GΩ) seals were obtained using unpolished patch pipettes pulled from borosilicate glass and filled with a high K^+/low Ca^{2+} solution containing: 140 mM KCl, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, and 696 mM glucose. The pH of the solution was adjusted to 7.0 with 5N KOH. Final K^+ concentration was 210 mM. With this solution, electrodes had impedances of 3-5 MΩ. Recorded signals were amplified with a Dagan Instruments 8900 Patch Clamp Amplifier equipped with a 1 GΩ feedback resistor. The bandwidth of the recording system was 10 kHz. Signals were displayed on a Nicolet 2090 Digital Oscilloscope and stored on floppy disks. Hard copies were obtained on a Houston Instruments 100 X-Y Plotter.

Preparation of Axolemma vesicles. Axolemma membranes were obtained from the Blue Crab (*Callinectes sapidus*), since this species was locally available and the walking leg nerves could be readily removed by breaking the leg joints and pulling the

nerves out. The method used was based on that described for spiny lobster (*Panularus argus*) axonal vesicles (Barnola et al., 1973) with minor modifications. In a typical preparation, the 8 walking legs and chelae neurons were dissected from 25 adult Blue Crabs. The neurons (wet weight 12 g) were minced with a Polytron rotary stainless steel knife mincer in 0.33 M sucrose, 2 mM $MgSO_4$, 10 mM Tris HCl, pH 7.4, at 4 °C (8 ml buffer per g wet weight tissue). This mixture was homogenized with Teflon-glass (10 strokes) at 500 rpm. The homogenate was centrifuged for 10 min at 3000 x g at 4 °C. The supernatant was retained and the pellet was rehomogenized in 40 ml 0.33 M sucrose, 2mM $MgSO_4$, 10 mM Tris HCl, pH 7.4, 4 °C, with a Teflon glass homogenizer (10 strokes). The rehomogenized pellet was centrifuged at 3000 x g for 10 min at 4 °C. The two supernatants were combined and the pellet was discarded. The supernatant was then centrifuged at 17,000 x g for 80 min at 4 °C. At this point, the supernatant was discarded and the pellet was recovered and homogenized (4 strokes) in 20 ml of 0.11 M sucrose, 2 mM $MgSO_4$, 5 mM Tris HCl, pH 7.4 at 4 C. This suspension was carefully layered on top of cellulose nitrate tubes containing discontinuous sucrose gradients consisting of 10 mM Tris HCl, pH 7.4 buffered sucrose solutions of 1.2 M, 1.0 M, 0.8 M, 0.6 M and 0.4 M (6 ml each). These tubes were centrifuged at 100,000 x g in a SW-28 rotor for 8 h at 4 °C. The dense white band at the 1.0-1.2 M sucrose interface was carefully removed and diluted dropwise to 0.33 M sucrose with

ice cold dH_2O. This mixture was centrifuged at 40,000 x g in a SW-28 rotor for 45 min at 4 °C. The pellets were recovered and rehomogenized (2 strokes by hand) in 20 ml of the homogenization buffer and stored at -78 °C. The 1.0-1.2 M sucrose fraction was used in all experiments. Characterization of this fraction included assay of [^{3}H]-STX binding to calculate receptor density, measuring the ability to retain a membrane potential as monitored by the fluorescence dye method using di-O-C_5 (Blaustein and Goldring, 1975). Protein concentration was determined by the bicinchonic acid (BCA) assay using bovine serum albumin as a standard (Redinbaugh and Turley, 1986). Enrichment of the axolemma fraction for the marker enzyme Na^+ - K^+ ATPase was determined by the method of Kilberg and Christensen (1979) where the released phosphate was quantitated spectrophotometrically (Fiske and Subbarow, 1925).

Preparation of Rat Brain Synaptosomes. Synaptosomes were prepared by a modification of the method of Gray and Whittaker (Gray and Whittaker, 1962). The brains were removed from 3 male Sprague-Dawley rats and homogenized in ice-cold 0.32 M sucrose containing 10 mM Tris HCl, pH 7.4 (10 ml/g tissue) with 10 strokes of teflon glass homogenizer (500 rpm). The resulting homogenate was sedimented at 1000 X g for 10 min at 4 °C. The supernatant was saved and the pellet was resuspended in 10 ml of 0.32 M sucrose and resedimented at 1000 X g for 10 min at 4 °C. The resulting supernatant was pooled with the first and

sedimented at 17,000 X g for 1 h at 4 °C. The supernatant was discarded and the pellet was resuspended in 8 ml of 0.32 M sucrose containing 10 mM Tris HCl, pH 7.4. This suspension was layered on to a stepwise gradient consisting of 6 ml of 1.2, 1.0, 0.8, 0.6, and 0.4 sucrose solutions buffered with 10 mM Tris HCl, pH 7.4, and sedimented at 100,000 X g for 105 min at 4 °C. The dense white band isolated at the 1.0-1.2 interface was used in all binding studies due to the presence of a high density of sealed vesicles (Michaelson and Whittaker, 1963). Characterization of this preparation for Na^+, K^+ ATPase enrichment, [^{3}H]-STX binding, and ability to maintain a membrane potential was performed as described earlier in the text for axolemma vesicles.

Electron microscopy. Axolemma vesicles and rat brain synaptosomes sucrose gradient fractions were monitored for the presence of sealed structures by negative stain electron microscopy. A solution of vesicles in 0.32 M sucrose buffered with 10 mM Tris HCl, pH 7.5 was allowed to adhere to a 400 mesh formvar carbon coated grid for 30 sec, blotted to remove excess solution, and then stained with a 2% solution of uranyl acetate for 30 sec. Excess stain was adsorbed with filter paper and air dried prior to examination in a Joel 100 CX electron microscope operated at 80 kV. Sealed vesicles and fragments were observed at a magnification of 165,000 X.

Radiolabeling *Androctonus austrialis hector* II with [^{125}I]. *Androctonus austrialis Hector* II (5 μg) was labeled by the lactoperoxidase-H_2O_2 and purified by gel filtration on a combination Sephadex G-15 and G-50 column as previously described (Rochat et al., 1977). The specific activity of the [^{125}I]-AaH II derivative was determined to be 460 Ci/mmol.

Radiolabeling ShN I with [^{125}I]. The chloramine-T method of iodination was used to incorporate [^{125}I] into the Tyr residues (Hunter and Greenwood, 1962). ShN I (0.2 mg) was dissolved in 0.25 ml of ice cold 20 mM Tris HCl, pH 8.64. To this solution 1.0 nmol of NaI carrier was added followed by a 10 μl aliquot of Na [^{125}I] (1 mCi). The iodination reaction was initiated by the addition of 10 μl of a 10 mM chloramine-T solution. Two additional 10 μl aliquots of the chloramine-T solution were added at 3 min intervals. The reaction was maintained at 0 °C for 30 min, at which point the entire reaction mixture was placed on a Sephadex G-25 column equilibrated in 50 mM $NaPO_4$, pH 7.5. The labeled protein eluted in the void volume of this column. The labeled protein was separated from unlabeled protein by reverse-phase HPLC using on a C_{18} column, with a gradient of 10% - 60 % acetonitrile into 0.1% TFA in 40 min at 2.1 ml/min. The unlabeled toxin eluted at 21 min into the gradient and the labeled material eluted at 22.3 min into the gradient.

Binding Experiments. [^{125}I]-ShN I and [^{3}H]-STX binding to synaptosomes and axolemma vesicles was measured by rapid filtration assay with glass fiber filters (Whatman GF/B). The standard binding medium consisted of 140 mM choline chloride, 50 mM Hepes adjusted to pH 7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml of bovine serum albumin. Samples were mixed and incubated for 30 min at 4 °C for [^{3}H]-STX and 37 °C for [^{125}I]-ShN I. Following 30 min incubation, the samples were diluted with 3.0 ml of ice cold wash buffer (163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 0.1 % bovine serum albumin) and immediately collected on glass fiber filters and washed with 3 X 3.0 ml of ice cold binding medium. The bound radioactivity was then determined by liquid scintillation counting for tritium or gamma emission for [^{125}I]. Counting efficiency for iodine and tritium was determined by the method of internal standards. Non-specific binding was measured in the presence of excess ligand (150 nM TTX or 10 μM ShN for axolemma vesicles and 250 μM ShN I for rat brain synaptosomes) and subtracted from the results. Binding measurements at each ligand concentration were done at least in duplicate, and the error measured for each data point was less than 2%.

Purification of Mono-specific Anti-ShN Antibodies. A *Staphylococcal* Protein A column was prepared by reacting 10 mg of Protein A with 3.0 ml of preswollen Affi-gel 15 for 4 h in

100 mM $NaCO_3$, 150 mM NaCl, pH 8.2 at 4 °C.. The remaining unreacted imido esters on the resin were treated with a 1.0 M solution of ethanolamine (Kodak Eastman, Kingsport, Tennessee) overnight at 4 °C. The binding capacity of the column was determined by monitoring the absorbance at 280 nm of the coupling buffer containing unreacted Protein A and determined to be greater than 3.2 mg Protein A per ml of resin. After being filtered through a Millipore (0.45 μm) antisera to ShN I was then passed over the column, and the column was washed extensively with PBS (25 mM $NaPO_4$, 140 mM NaCl, pH 7.8) until the A_{280} returned to 0. The IgG fraction was then eluted from the column with a buffer consisting of 0.58 % acetic acid and 140 mM NaCl. The eluted IgG fraction was immediately neutralized with a solution 1.5 M Tris base and 140 mM NaCl to pH 7.8.

Radioimmunoassay for Crossreactivity. Radioimmunoassay was by the method of Chandler et al. (1984). A 96 well microtiter plate was coated with a 25 μl of 0.1 mg/ml solution of Protein A dissolved in PBS. The microtiter plate was incubated for 1 h at room temperature and then washed with 3 X 100 μl of PBS. The IgG fraction was diluted with PBS to 1/2000 of serum volume and 25 μl was plated out in each well. The plate was then incubated for 1.5 h at room temperature and subsequently washed with 5 X 100 μl of RIA buffer (25 mM $NaPO_4$, 140 mM NaCl, 0.05% Tween 20, and 0.3% BSA at pH 7.8). To each well was added 25 μl of a

1/100 dilution of [^{125}I]-ShN I (approximately 4000 cpm), and 25 μl of the appropriate dilution of unlabeled toxin sample. Following an incubation of 1.5 h at room temperature, each well was washed with 5 X 100 μl of RIA buffer. The wells were then punched out and read directly on a Beckman 5500 gamma counter. Each toxin dilution was run in triplicate and the data represents an average of the values obtained where the standard error measured for each point was less than 3%.

Results

Intracellular Recording. Preliminary electrophysiological studies using whole cell patch clamping of lobster (*Panularus argus*) olfactory somas indicate that ShN I delays the inactivation of the Na^+ current (Figure 2:1). In control experiments, application of a stimulus resulted in an initial Na^+ current which rapidly returned to resting values (Figure 2:1A). Following application of ShN I, the Na^+ current was observed to be prolonged with a slow return to resting values (Figure 2:1B). Application of TTX to the external saline solution abolished the effects observed with ShN I. TTX immediately blocked the inward Na^+ currents (data not shown). These results are consistent with those observed with other polypeptides which bind to the Na channel and delay inactivation (for review see Catterall, 1980)

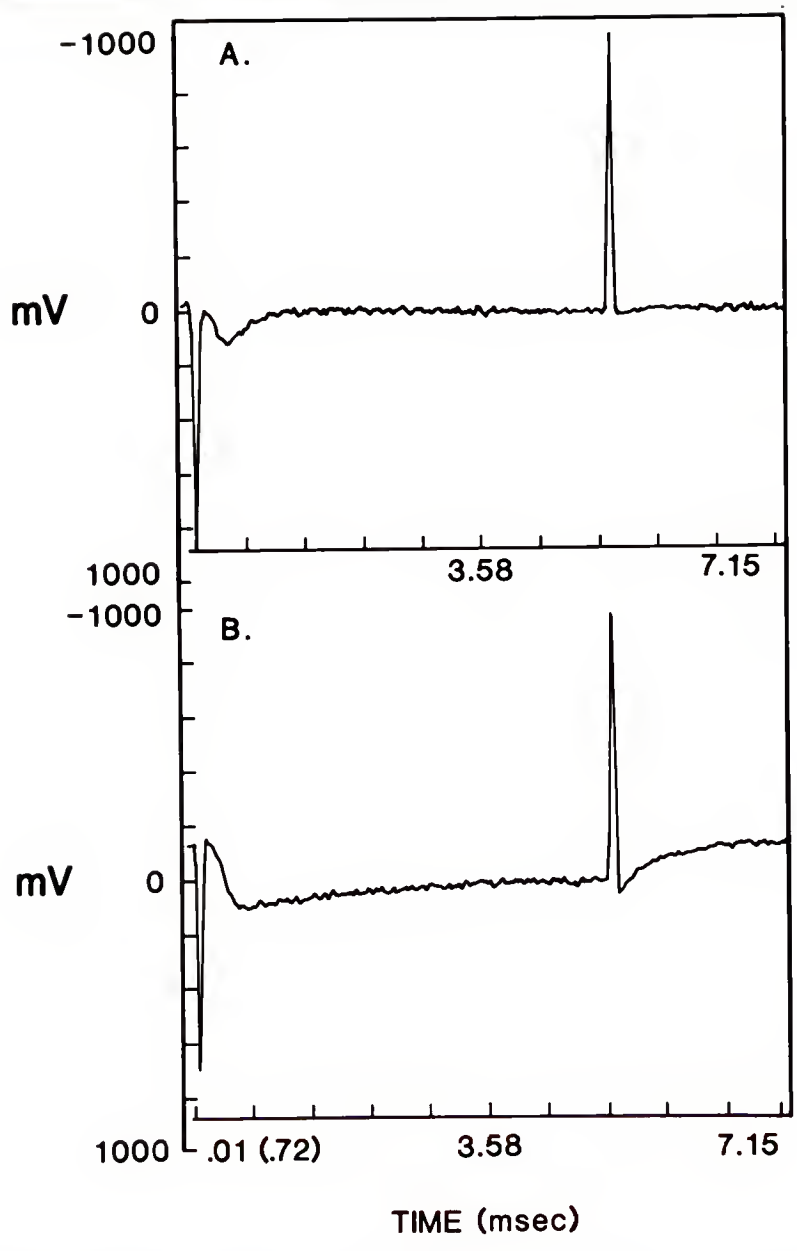


Figure 2:1: Electrophysiological effects of ShN I. Whole cell patch clamping was used to monitor the effects of ShN I. (A) Control recording following a 2 mV stimulus. (B) Recording of same cell following application 100 μl of 1 μM ShN I solution. Final toxin concentration in the bath was 100 nM.

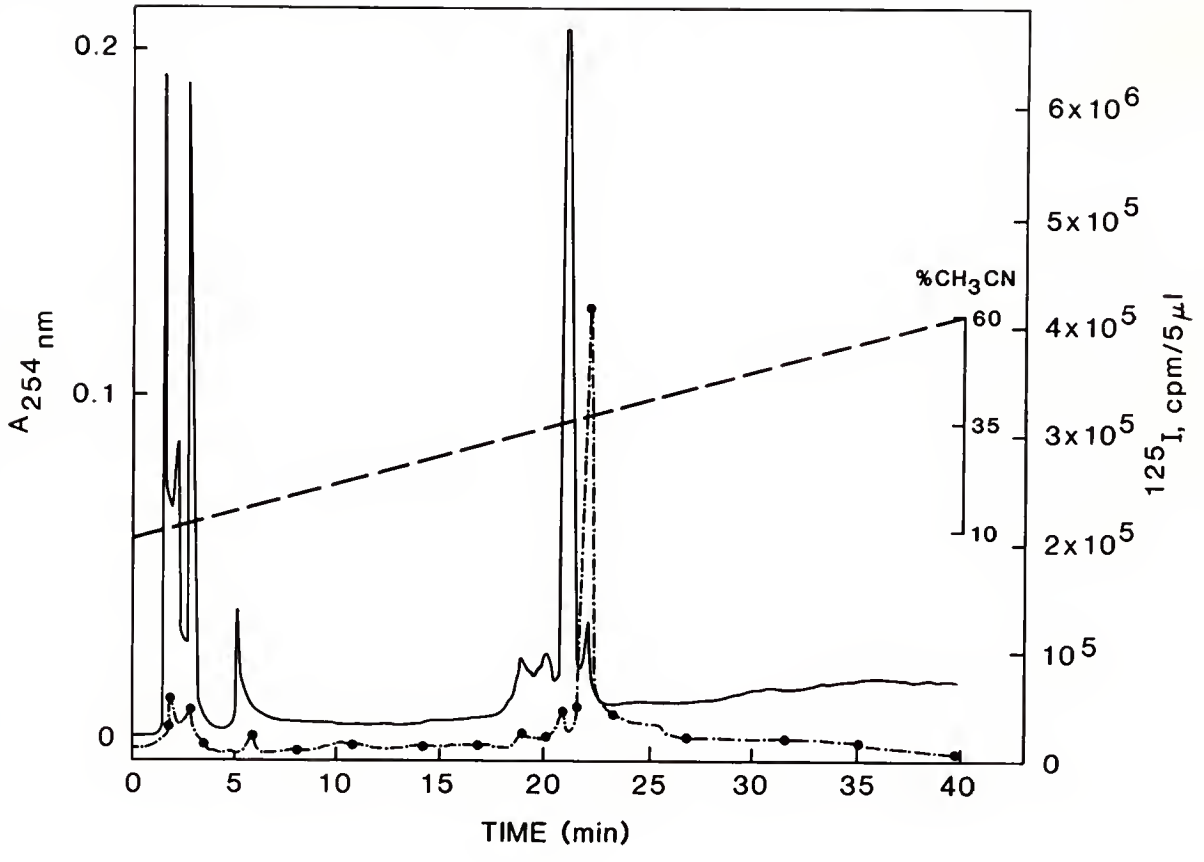


Figure 2:2: Purification of [^{125}I]-ShN I. ShN I (300 μg) was iodinated by the chloramine-T method at 0 °C. Following the iodination reaction, the reaction mixture was immediately filtered on a Sephadex G-25 column (0.75 X 21 cm) equilibrated with 50 mM $NaPO_4$, pH 7.5. The void volume peak was collected and rechromatographed on reverse-phase HPLC using a C_{18} column. The gradient consisted of 10-60% acetonitrile into 0.1% TFA in 40 min at 2.0 ml/min. The chromatography was followed by automatic recording of absorbance at 254 nm (—) and by measurement of radioactivity of a 10 μl aliquot removed from each collected fraction monitored in a gamma counter (–•–).

Preparation of Radiolabeled Derivative. ShN I was iodinated by the chloramine-T method (Hunter and Greenwood, 1962). Specific activities varied between 50 and 800 Ci/mmol depending upon the inclusion of cold ^{127}I into the reaction mixture. Purification of the iodinated toxin involved an initial desalting step on a Sephadex G-25 column followed by reverse-phase HPLC. This final purification step on reverse-phase HPLC was necessary to isolate the [^{125}I]-ShN I from the unreacted ShN I. The separation data is shown in Figure 2:2. Manipulation of the gradient of acetonitrile increased the retention time of the derivatized material allowing it to be easily separated from the native toxin. The protein concentration was then determined through measurement of the intrinsic absorption at 280 nm due to the presence of two Tyr and one Trp residue (A^{280} of 1% solution 13.92). Following determination of the protein concentration the iodinated toxin was lyophilized and redissolved in the standard binding buffer which contained 1mg/ml BSA (see "Experimental Methods").

Pharmacological properties of the [^{125}I]-ShN I derivative determined the LD_{50} on fiddler crab to be approximately 3.2 μg/kg (native toxin LD_{50} = 2.92 μg/kg). Thus, the iodinated derivative possessed greater than 90% of the activity of the native toxin.

Characterization of Crab Axolemma Vesicles and Rat Brain Synaptosomes. Blue Crab walking leg axolemma vesicles and rat

brain synaptosomes were prepared as described previously. The purified axolemma and synaptosome preparation were then tested with [^{3}H]-STX for the presence of voltage sensitive sodium channels. The binding capacity for [^{3}H]-STX was approximately 14 pmol/mg for axolemma vesicles and 1.8 pmol/mg for the synaptosomes. The ability of the axolemma vesicles as well as the synaptosomes to maintain a membrane potential was determined with the voltage sensitive dye di-O-C_5, (Blaustein and Goldring, 1975). The fluorescence properties of the dye could be modified by changing the K^+ concentration or by adding veratridine in Na^+ buffer. Lysis of the vesicles with a detergent such as Triton X-100 caused the return of fluorescence back to baseline values. Enrichment for the marker enzyme Na^+,K^+ ATPase was approximately 12 times that of the starting homogenate for axolemma vesicles and 6 fold enrichment for synaptosomes. Vesicle preparations were also monitored by electron microscopy (Figure 2:3). Sealed vesicles (Figure 2:3A) were easily distinguished from the unsealed fragments (Figure 2:3B) found in fractions taken from the lower density bands of the discontinuous sucrose gradient.

[^{125}I]-ShN I Binding to Axolemma Vesicles. Initial experiments were designed to detect saturable binding of [^{125}I]-ShN I to sites with a K_D of 1-1000 nM. In these experiments, the vesicles were incubated in the presence of increasing concentrations of [^{125}I]-ShN I (Figure 2:4, left O).

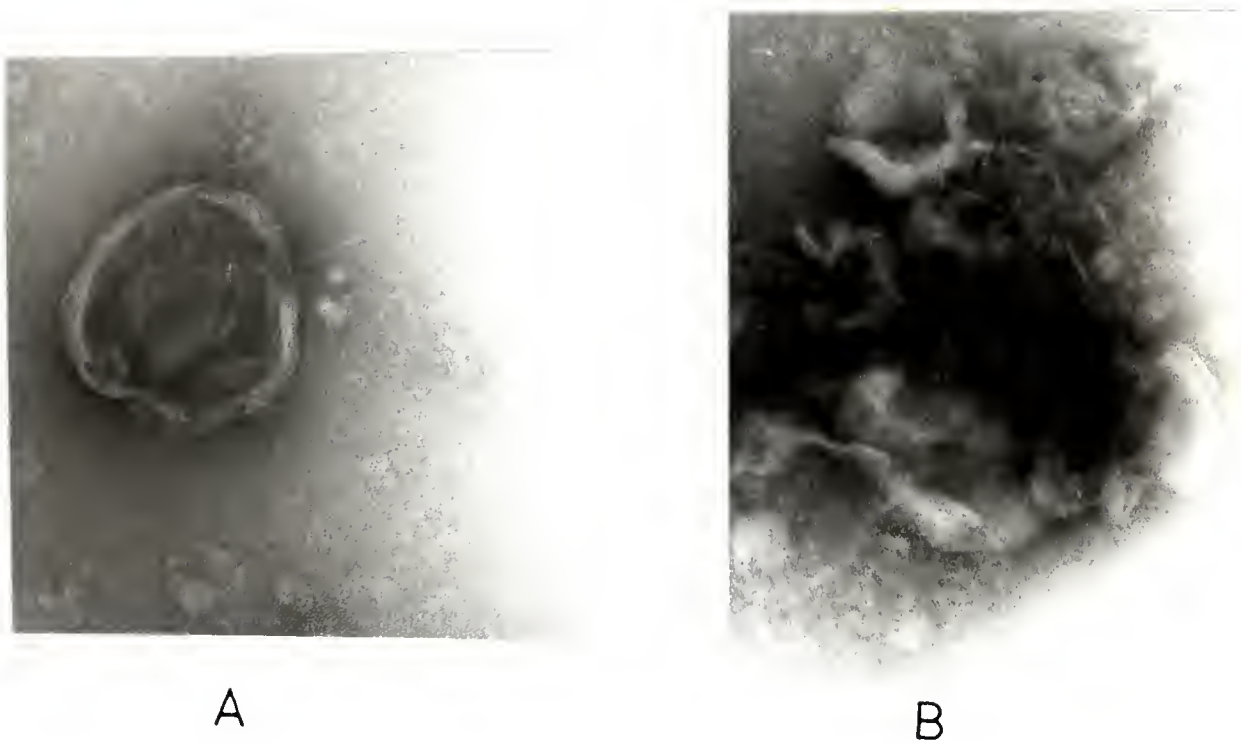


Figure 2:3: Electron Micrographs of Sealed Axolemma and Synaptosome Vesicles. A, axolemma vesicles isolated from discontinuous sucrose gradient centrifugation. Magnification x 125,000. B, unsealed axolemma vesicles isolated from the 0.6 - 0.8 M sucrose interface.

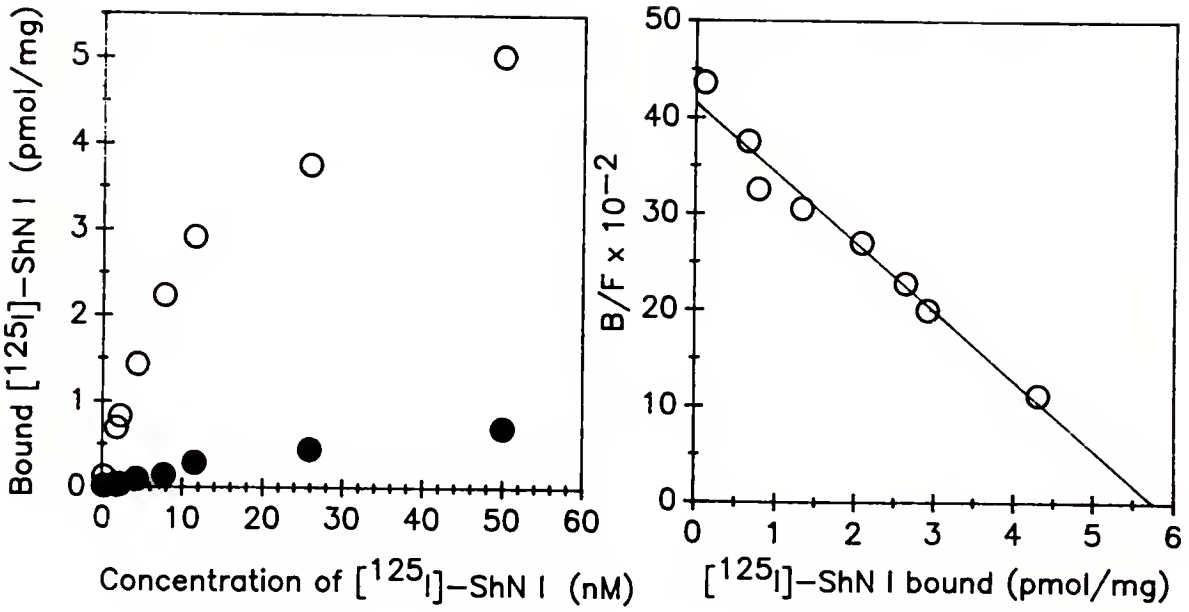


Figure 2:4: Scatchard Analysis of ShN I binding to axolemma vesicles. *Left*, axolemma vesicles were incubated with increasing concentrations of [^{125}I]-ShN I as indicated on the *abscissa* in standard binding media (O) or in standard binding media containing 10 μM ShN I (saturation concentration) (●). Bound [^{125}I]-ShN I was then measured as described under "Experimental Procedures". *Right*, specific binding, calculated as the difference between binding in the presence and absence of 10 μM ShN I, is plotted as a Scatchard Plot.

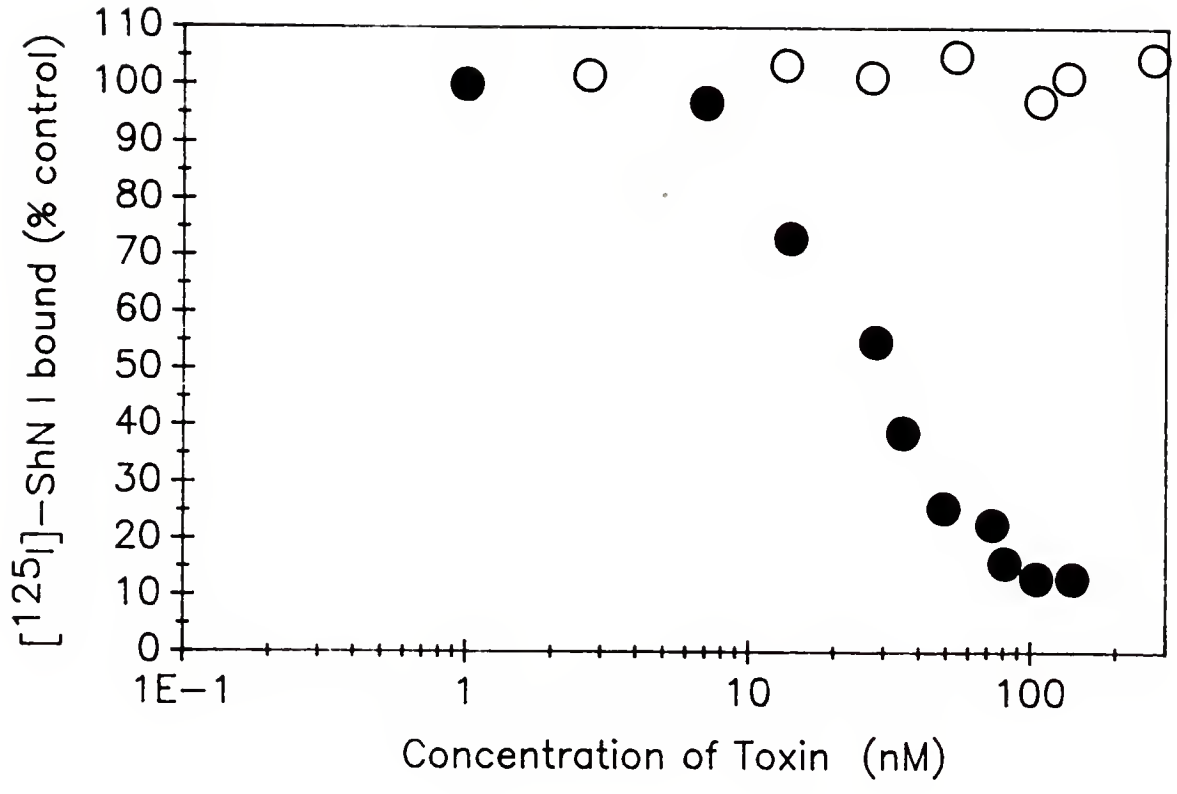


Figure 2:5: Competition between [^{125}I]-ShN I and ShN I or As II for binding to axolemma vesicles. [^{125}I]-ShN I (0.5 nM) was incubated with axolemma vesicles (0.5 mg of protein per ml) and increasing concentrations of ShN I (●) or As II (O) in the standard binding media. Following 30 min incubation at 37 °C, the radioactivity bound was then determined as described under "Experimental Methods".

Nonspecific [^{125}I]-ShN I binding was measured in the presence of an excess of unlabeled ShN I (10 μM) (Figure 2:4, left ●). Saturable binding, which is defined as the difference between specific and nonspecific binding curves in Figure 2:4, is presented in the form of a Scatchard plot in Figure 2:4 (right). The linearity of the Scatchard analysis (Scatchard, 1949) indicates a single class of receptors with K_D of 14 nM and a binding capacity of 5.8 pmol/mg.

[^{125}I]-ShN I versus ShN I Binding to Axolemma Vesicles. Axolemma vesicles were incubated with 0.5 nM [^{125}I]-ShN I and increasing concentrations of unlabeled ShN I from 1 to 500 nM. Assuming that there are saturable binding sites for ShN I, these sites should be increasingly occupied by unlabeled ShN I, resulting in decreased [^{125}I]-ShN I binding. The results of these experiments (Figure 2:5) determined that unlabeled ShN I inhibits greater than 85% of [^{125}I]-ShN I binding with 50% maximal inhibition at 25 nM. These results suggest a saturable ShN I receptor exists in the axolemma vesicles with a $K_{0.5}$ of approximately 25 nM. In these experiments, nonsaturable binding was determined in the presence of 10 μM ShN I and was subtracted from the results.

Recently, long anemone toxins have been divided into two classes according to sequence homology (Schweitz et al., 1985; Kem, 1988). As II is classically used as the representative type 1 long anemone toxin in competition binding experiments

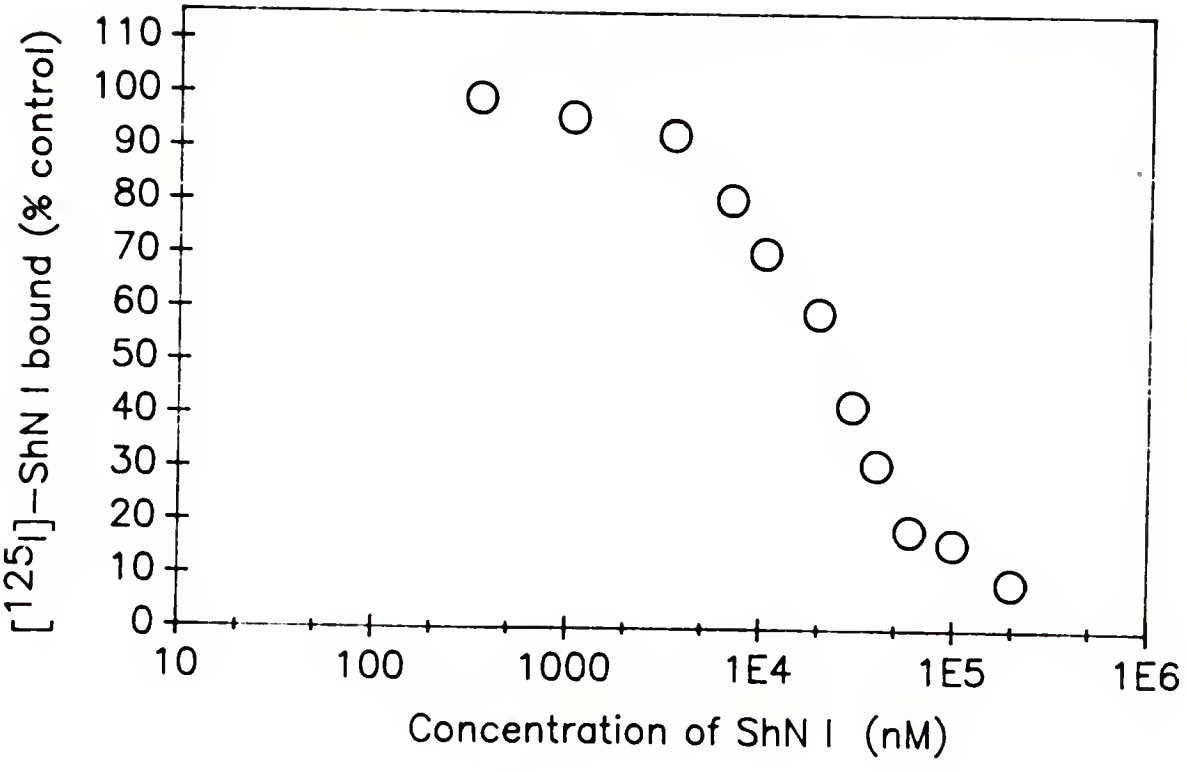


Figure 2:6: Competition between [^{125}I]-ShN I and ShN I for binding to rat brain synaptosomes. [^{125}I]-ShN I (10 nM) was incubated with rat brain synaptosomes (1 mg protein per ml) and increasing concentrations of ShN I. Following 30 min incubation at 37 °C, the radioactivity bound was then determined as described under "Experimental Methods".

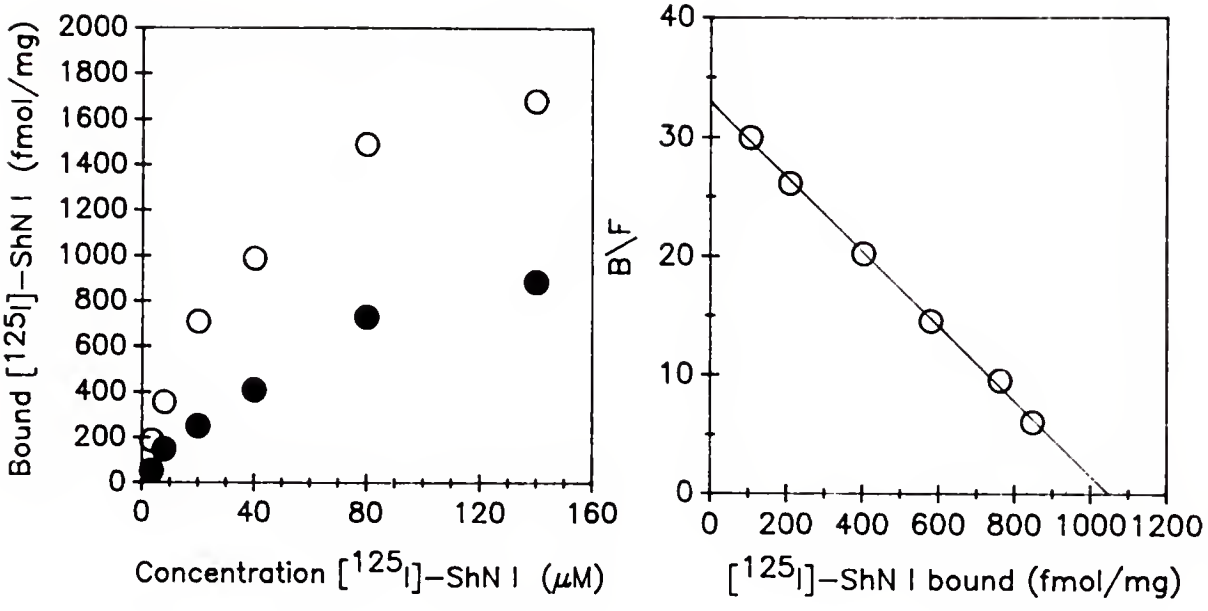


Figure 2:7: Scatchard analysis of [^{125}I]-ShN I binding to rat brain synaptosomes. *Left*, synaptosomes were incubated with increasing concentrations of [^{125}I]-ShN I as indicated on the *abscissa* in standard binding media (○) or in standard binding media containing 250 μM ShN I (●). Bound [^{125}I]-ShN I was then determined as described under "Experimental Methods". *Right*, specific binding was then calculated as the difference between binding with and without 250 μM ShN I, is plotted in the form of a Scatchard plot.

with scorpion toxins and other sea anemone toxins (Catterall and Beress, 1978; Vincent et al., 1980). Experiments were carried out, as described above for unlabeled ShN I, to determine the ability of As II (Figure 2:5, O) to displace [^{125}I]-ShN I on the axolemma vesicles. As II was unable to displace the [^{125}I]-ShN I in these experiments over a concentration range from 10 nM to 500 nM. Axolemma vesicles were selected for these studies because of the specificity which ShN I exhibited in binding to these versus rat brain synaptosomes. Similar results for the competition of *Heteractis paumotensis* toxins with [^{125}I-As V on rat brain synaptosomes have been reported (Schweitz et. al. 1985). However, ShN I exhibits a much lower toxicity to mammals than the Hp toxins. Thus, these competition experiments were not attempted on rat brain synaptosomes. Our results along with those of Schweitz et al. (1985) suggest the possibility of a separate anemone type 2 toxin receptor on both axolemma vesicles and synaptosomes.

Binding of ShN I to Rat Brain Synaptosomes. Following the same logic as used for the axolemma vesicles, a class of receptors was suspected to exist with a substantially higher K_D value due to the much lower LD_{50} that ShN I exhibited on mice. Thus, synaptosomes were incubated with 10 nm [^{125}I]-ShN I and increasing concentrations of unlabeled ShN I from 50 to 500,000 nM. Assuming the presence of a saturable receptor for the ShN I toxin, these sites should be increasingly occupied by the

unlabeled ShN I resulting in the same decrease of [^{125}I]-ShN I binding observed in the axolemma vesicles. The results of these experiments (Figure 2:6, O) show that unlabeled ShN I displaces greater than 90% of the [^{125}I]-ShN I with 50% maximal inhibition observed at 26,000 nM, suggesting the presence of saturable receptor with a K_D of approximately 26,000 nM.

The presence of a saturable receptor was also determined through experiments where the concentration of [^{125}I]-ShN I was varied. In these experiments, total ShN I binding is measured in incubation mixtures containing increasing concentrations of [^{125}I]-ShN I (Figure 2:7, left O). Nonsaturable [^{125}I]-ShN I binding was determined in the presence of excess unlabeled ShN I (250 μM) (Figure 2:7, left ●). The difference of these two curves represents the saturable binding component and is presented in the form of a Scatchard Plot (Figure 2:7, right). The Scatchard analysis indicates the presence of a single class of receptor sites for [^{125}I]-ShN I with a K_D = 31 μM and a B_{max} of 1.08 pmol/mg of protein.

Characterization of ShN I Receptor. Investigation of the synaptosome toxin receptor was also probed using the α-scorpion toxin, [^{125}I]-AaH II. In these experiments, synaptosomes were incubated with 0.02 nM [^{125}I]-AaH II and increasing concentrations of AaH II (Figure 2:8, O), As II (Figure 2:8, ●), or ShN I (Figure 2:8, Δ). The unlabeled AaH II displaced the [^{125}I]-AaH II with 50% maximal inhibition observed at 450 pM.

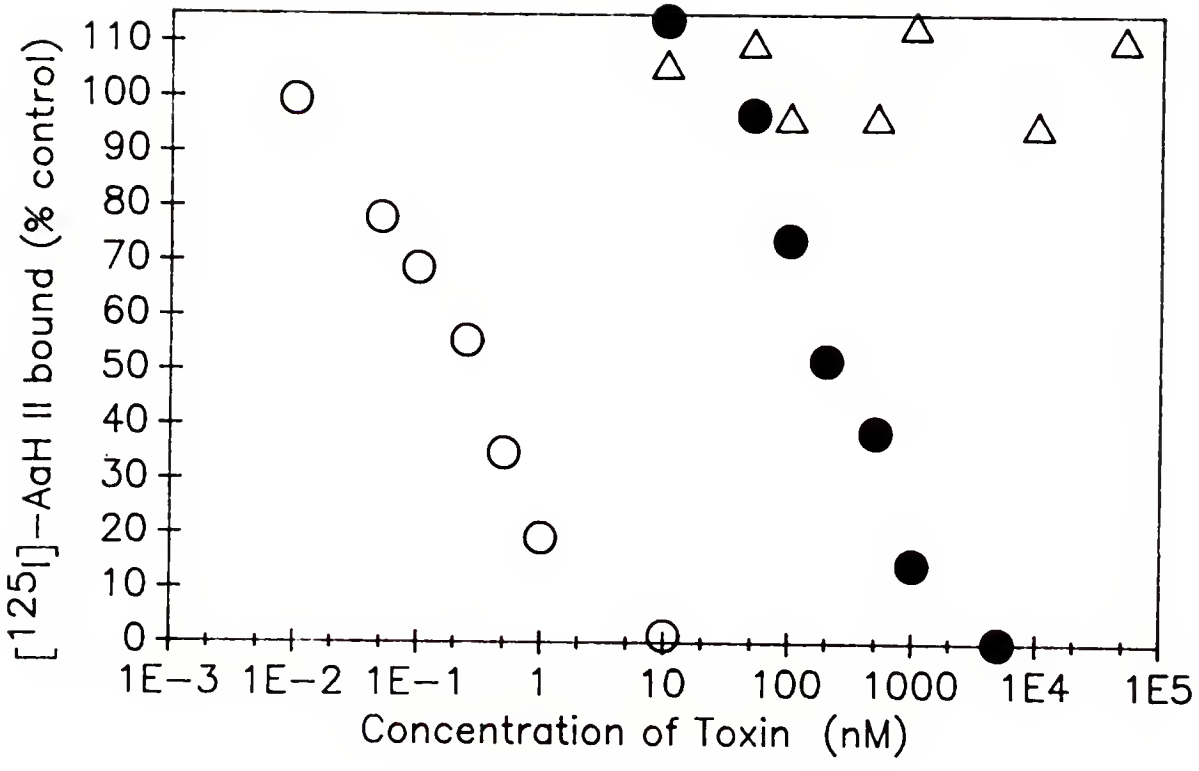


Figure 2:8: Competition between [^{125}I]-AaH II and ShN I or As II binding to rat brain synaptosomes. [^{125}I]-AaH II (0.02 nM) was incubated with rat brain synaptosomes (1 mg protein per ml) and increasing concentrations of AaH II (O), ShN I (Δ) or As II (●) in standard binding media. Following 30 min incubation at 37 °C, the radioactivity bound was determined as described under "Experimental Methods".

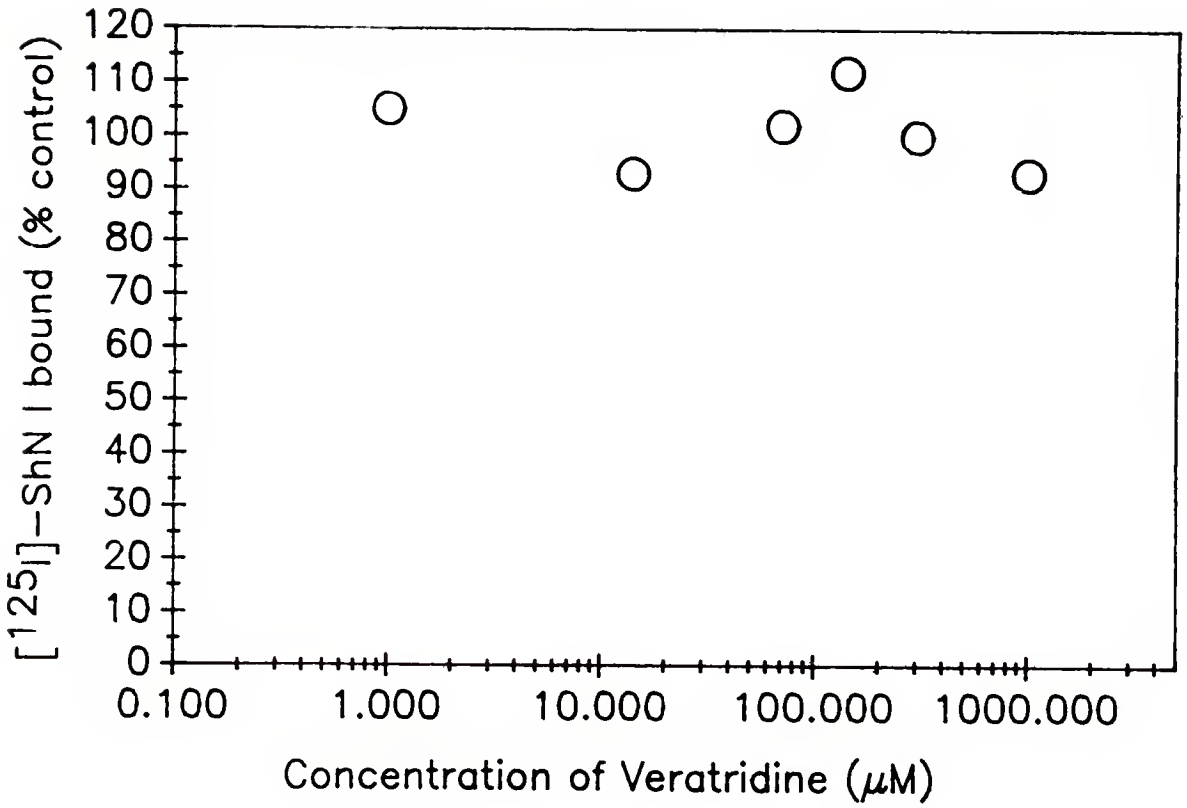


Figure 2:9: Enhancement of [^{125}I]-ShN I binding by veratridine. Axolemma vesicles (0.5 mg protein per ml) were incubated with 0.5 nM [^{125}I]-ShN I, in the presence of increasing concentrations of veratridine in standard binding media. Following 30 min incubation at 37 °C, the bound radioactivity was determined as described under "Experimental Methods".

Similarly, As II displaced the [^{125}I]-AaH II with 50% maximal inhibition at 300 nM; however, ShN I was unable to displace any of the [^{125}I]-AaH II from its receptor. Competition assays with the [^{125}I]-AaH II on axolemma vesicles were not attempted due to the low toxicity which this toxin has on crustaceans. These results suggested that the ShN I was binding to a separate receptor population in the synaptosomes.

Classical site III polypeptides have been shown to interact cooperatively with site II lipophilic toxins such as BTX and veratridine (Catterall, 1977; Ray et al., 1978). Alpha-scorpion toxins enhance the activation of the Na^+ channels by these compounds through an allosteric mechanism (Catterall, 1977b). Thus, the binding of scorpion toxin is enhanced in the presence of these compounds in both synaptosomes and neuroblastoma cells (Catterall, 1977a; Ray et al., 1978). In order to determine if the binding of [^{125}I]-ShN I is affected by these site II compounds, experiments were carried out exclusively in choline substituted buffers in order to eliminate any effect of membrane potential dependent binding. The increase in binding of [^{125}I]-ShN I was measured with increasing concentrations of veratridine (Figure 2:9). No increase in binding of [^{125}I]-ShN I was detected up to a concentration of 1000 μM veratridine in either of the synaptosomal or axolemmal preparations. These results indicate that there is little or no heterotrophic cooperativity between veratridine and ShN I.

Site III binding polypeptides have all shown some type of membrane potential dependent binding (Catterall et al., 1976; Catterall, 1977; Ray et al., 1978). However, scorpion toxin [e.g., Lqq V] binding is affected much more severely than anemone toxins such as As II (Catterall and Beress, 1978; Ray et al., 1978; Vincent et al., 1980). In order to test the effects of membrane potential on binding, incubations were carried out in buffers containing 130 mM KCl which completely depolarizes the synaptosomes (Blaustein and Goldring, 1975), or 130 mM NaCl with gramicidin D, a Na^+ ionophore, which completely depolarizes the synaptosomes (Blaustein and Goldring, 1975). These results are presented in Table 2:1. Neither the incubation in 130 mM KCl nor the Na^+/gramicidin experiment showed any significant reduction in the binding of [^{125}I]-ShN I to either synaptosomes or axolemma vesicles. Osmotic lysis of the vesicle preparations is expected to result in depolarization (Blaustein and Goldring, 1975). Lysis of either preparation in dH_2O at 0 °C prior addition of [^{125}I]-ShN I also resulted in no apparent reduction of binding of [^{125}I]-ShN I (Table 2:1). Therefore, we conclude that this particular receptor class is not dependent on the presence of a membrane potential.

Immunocrossreactivity of anemone toxins. The Protein A purified anti-ShN I antibody was diluted to 1/2000 the serum volume. The antibody was immunoreactive with the labeled [^{125}I]-ShN I derivative as shown in Figure 2:10 (●). The standardization

Table 2:1. Effects of depolarization on [^{125}I]-ShN I binding

Conditions	% [^{125}I]-ShN I bound
Experiment I	
A. Axolemma	
130 mM NaCl	89
130 mM KCl	94
130 mM NaCl, gramicidin D (10 μg/ml)	92
B. Synaptosomes	
130 mM NaCl	99
130 mM KCl	104
130 mM NaCl, gramicidin D (10 μg/ml)	103
Experiment II	
A. Axolemma normal	100
Lysed	89
B. Synaptosomes normal	100
Lysed	104

In Experiment I, [^{125}I]-ShN I binding was measured as described under "Experimental Methods" in solutions in which the choline chloride (130 mM) present in the standard incubation medium was replaced either with NaCl (130 mM), KCl (130 mM), or with NaCl (130 mM) containing 10 μg/ml gramicidin D. The results are presented as the percentage of the binding versus the binding measured in the standard choline chloride (130 mM) medium. In Experiment II, the binding was also determined as described under "Experimental Methods", using either synaptosomes or axolemma vesicles which had been lysed by prior incubation in dH_2O or with normal preparations. The nonspecifically bound [^{125}I]-ShN I was determined in the presence of either 10 μM or 250 μM ShN I for axolemma and synaptosomes, respectively, and subtracted from each value prior to any calculation of percent bound.

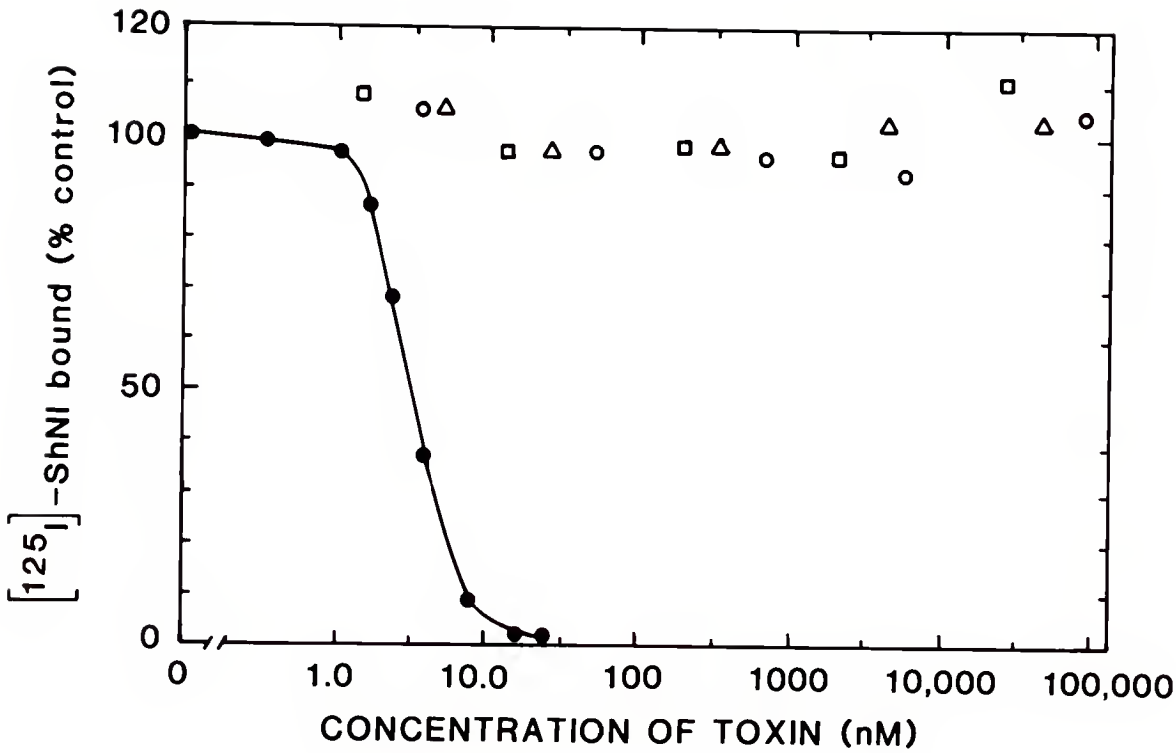


Figure 2:10: Competition between [^{125}I]-ShN I and different sea anemone toxins for association with ShN I antibodies. The antiserum was at a dilution of 1/2000. (Ordinate) Ratio of bound [^{125}I]-ShN I in the presence of increasing concentrations of sea anemone toxin to total [^{125}I]-ShN I bound (without any unlabeled toxin present). (●) ShN I, (O) As II, (△) Bol II, and (□) Condyl III.

curve obtained with increasing concentrations of unlabeled ShN I determined the $K_{0.5}$ to be 4.7 nM. This anti-ShN I antibody was tested for immunocrossreactivity with several other sea anemone toxins. As shown in Figure 2:10, concentrations up to 10 μM for Bol II (Δ), Condyl III ($\square$), and As II (O) failed to displace any of the counts specifically bound to the anti-ShN I antibody.

Discussion

Sea anemone polypeptide neurotoxins bind specifically voltage-sensitive Na^+ channels in synaptosomes, neuroblastoma cells, and rat skeletal myoblasts (Catterall, 1976; De Barry et al., 1977; Jacques et al., 1978; Catterall and Beress, 1978; Romey et al., 1980; Vincent et al., 1980; Schweitz et al., 1981; Schweitz et al., 1985). These toxins cause a delay in the inactivation of the Na channel similar to the effects elicited by α-scorpion toxins (Koppenhofer and Schmidt, 1968; Narahashi et al., 1969). Anemone toxins typically bind with dissociation constants approximately two to three orders of magnitude greater than α-scorpion polypeptide neurotoxins (Catterall and Beress, 1978; Vincent et al., 1980; Schweitz et al., 1981). Several of these sea anemone polypeptides have very unusual species selectivity in that they have a much lower LD_{50} for representative crustacean species than for a representative mammalian species (Table 2:2). Measurement of the K_D for these toxins showing very little mammalian toxicity has been difficult

using the classical model, rat brain synaptosomes (Schweitz et al., 1981; Schweitz et al., 1985). The toxin which we isolated from the Caribbean sea anemone *Stichodactyla helianthus* was extremely toxic to crabs with an LD_{50} of 2.98 μg/kg and showed very little mammalian activity when injected intraperitoneally (LD_{50} > 15,000 μg/kg). We have determined that the electrophysiological effects of this toxin are similar to those of other anemone toxins as well as α-scorpion toxins. Thus, we have used a crustacean vesicle preparation derived from the walking leg nerves of Blue Crabs to measure the crustacean K_D as well as rat brain synaptosomes. Characterization of both of these preparations by several procedures including: enrichment of marker enzymes, binding of [^{3}H]-STX, electron microscopy, and membrane potential measurement showed both preparations contain sealed vesicles with voltage-dependent Na^+ channels present. Using the iodinated derivative of ShN I, a single class of saturable binding sites was determined in both rat brain synaptosomes and crab axolemma vesicles. However, the K_D for crab axolemma vesicles is more than three orders of magnitude lower (14.6 nM) than that determined for rat brain synaptosomes (31,000 nM). The maximal binding capacity of the axolemma vesicles and synaptosomes for [^{3}H]-STX was approximately 14.0 pmol/mg of protein and 1.7 pmol/mg of protein, respectively. Whereas the maximal binding capacity for [^{125}I]-ShN I on axolemma vesicles was approximately 5.8 pmol/mg of protein, and 1.08 pmol/mg of protein on synaptosomes. Thus, there are

approximately two STX binding sites per ShN I binding site in this preparation. Interestingly, neuroblastoma cells have been determined to contain 2.8 TTX receptors per scorpion toxin receptor (Catterall and Morrow, 1978).

We have shown that a polyclonal antibody prepared against ShN I had no cross reactivity with any of the type 1 anemone toxins. This supports the previous results by Schweitz et al. (1985) with Hp toxins. The lack of immunocrossreactivity despite the significant similarities in structure supports the separation into two different types of anemone toxins.

Binding properties of another Stichodactylid neurotoxin (Hp II) have been investigated in ion flux experiments using rat skeletal myoblasts (Schweitz et al., 1985). In the same report, they have reported competition binding results on rat brain synaptosomes using [^{125}I]-As V, [^{125}I]-AaH II and [^{125}I]-TiTx τ versus *Heteractis paumotensis* toxins. Their results suggested the possibility of separate anemone toxin binding sites, however, no characterization beyond basic competition experiments was presented. We have determined that As II is unable to displace [^{125}I]-ShN I in axolemma vesicles and ShN I is unable to displace [^{125}I]-AaH II in synaptosomes, but As II and unlabeled AaH II do displace the [^{125}I]-AaH II. These studies indicate that two different anemone toxin receptors are present in both crustacean and mammalian species.

Classical studies on site III polypeptide toxins have characterized this receptor as being membrane potential

dependent in its binding properties (Catterall et al., 1976; Catterall, 1977; Ray and Catterall, 1978; Ray et al., 1978). Depolarization of synaptosomes caused 89% reduction of binding for scorpion toxins as well as large increase of the K_D for the receptor-ligand complex (Ray et al., 1978). Sea anemone toxins do not show the same membrane potential dependence as the scorpion toxins in binding to either neuroblastoma cells or synaptosomes (Catterall and Beress, 1978; Vincent et al., 1980). We investigated the requirement for membrane potential dependent binding of ShN I in both synaptosomes and axolemma vesicles. Using several different methods to cause depolarization, we observed no reduction in the binding of [^{125}I]-ShN I in either preparation. Osmotic lysis of the vesicles has also been used previously to depolarize vesicles (Blaustein and Goldring, 1975). Assuming that there existed a 1:1 mixture of right-side-out and inside-out vesicles, an increase of overall [^{125}I]-ShN I bound to the vesicles due to more accessible receptor sites may be expected. We observed only slight increase in binding of [^{125}I]-ShN I. This low increase was most likely the result of re-sealing of the vesicular compartment following the osmotic shock. Studies on accumulation of $^{42}K^+$ on synaptosomes subjected to hypotonic osmotic lysis have shown that a significant fraction do re-seal following this treatment and are able to accumulate $^{42}K^+$ against a concentration gradient (Blaustein and Goldring, 1975).

Another property exhibited by site III polypeptide toxins is the cooperative interaction with the site II lipophilic toxins such as BTX and veratridine (Ray et al., 1978; Jacques et al., 1978; Romey et al., 1980; Tamkun and Catterall, 1981). Binding experiments to rat brain synaptosomes with labeled scorpion toxin [^{125}I]-Lqq V in the presence of BTX resulted in a 4 fold increase of [^{125}I]-Lqq V above control (Ray et al., 1978). In the same report, veratridine resulted in a 2.3 fold increase of [^{125}I]-Lqq V. Sea anemone toxins and veratridine have been shown to have a synergistic action on action potential in neuroblastoma cells (Jacques et al., 1978; Romey et al., 1980). Thus, if ShN I was binding at the same site as Lqq V, it would be expected to observe the same or similar type of increased binding in the presence of veratridine. Our results have determined that veratridine concentrations up to 1000 μM had no apparent effect on the binding of [^{125}I]-ShN I in either synaptosomes or axolemma vesicles. Interestingly, the toxin Sg I in the presence of veratridine resulted in only slight stimulation of $^{22}Na^{+}$ flux in neuroblastoma cells at concentrations greater than 10 μM, and Hp II, another toxin of the same sea anemone family Stichodactyilidae, showed similar stimulation of $^{22}Na^{+}$ flux in the presence of veratridine with an ED_{50} of 30 μM (Schweitz et al., 1981; Schweitz et al., 1985). The stimulation of $^{22}Na^{+}$ flux in the presence of veratridine observed for both of these toxins in neuroblastoma cells was much lower than that observed for the anemone toxins belonging

to the family Actiniidae. This provides evidence that the cooperativity of site II toxins with site III scorpion and sea anemone polypeptides is not experienced or only very slightly experienced by the Stichodactylid toxins.

The discovery of a second class of long sea anemone toxins has now lead to the discovery and characterization of a new receptor class on the Na channel. This particular receptor may be contiguous with the AaH II receptor site (Schweitz et al., 1985); however, the toxin Hp II was unable to displace [^{125}I]-AaH II (Schweitz et al., 1985). Similarly, ShN I was unable to displace this radiolabeled AaH II from its receptor on rat brain synaptosomes. This may actually implicate even another receptor site and possibly subdivide the class II long sea anemone toxins.

Comparison of the sequences of the reported class II long sea anemone toxins shows that they all possess a remarkable amount of sequence homology. Recalling the markedly diverse species selectivity that these toxins exhibit (Table 2:2), differences in the primary sequences among these toxins are few and provide the basis for determining which residues may be involved in conferring this selectivity. We have established and characterized two different binding systems for ShN I, to serve as models in preparation for our study of structure-function relationships in the ShN I molecule.

Table 2:2. Pharmacological Properties of Purified Sea Anemone Toxins

Toxin	Toxicity (μg/kg) Crab LD_{50}[a]	Toxicity (μg/kg) Mice LD_{50}[b]	Rat Brain Synaptosome K_D (nM)
ShN I	3	>15,000	31,000
As I	8	4,000	7,000
As II	8	100	150
As III	14	>18,000	>10,000
As V	20	19	50
Hp III	26	53	300
Sg I	28	>2,000	>10,000
Hp II	40	4,200	>100,000
Ax I (*Anthopl.* A)	44	66	120
Ax II (*Anthopl.* B)	160	8	35
Hp IV	230	40	10,000
Hm III	820	2	---

[a]Injection into intrahemocoelic space.

[b]Intraperitoneal injection.

(Taken from Kem, 1988).

CHAPTER III

CHEMICAL SYNTHESIS OF THE MAJOR NEUROTOXIN FROM THE SEA ANEMONE *STICHODACTYLA HELIANTHUS*

Introduction

Technological advances in the assembly of the peptide chain have made the synthesis of a fifty residue peptide feasible. For example, the development of a more acid stable resin incorporating the phenylacetamidomethyl linker group has decreased the amount of chain loss during synthesis (Mitchell et al., 1978). Also improvements have been made in the process of solvating the resin which helps to minimize the aggregative properties of the resin bound peptide (Meister and Kent, 1983). Highly reactive preformed symmetric anhydrides have been used to increase the coupling efficiency (Hagenmaier and Frank, 1972). Another significant improvement was made in the development of a quantitative technique to monitor the efficiency of coupling at each step (Sarin et al., 1981). Incorporating these improvements into an automated system has allowed peptides of greater than 100 residues to be synthesized (Merrifield, 1983; Clark-Lewis et al., 1986).

The classical synthesis of ribonuclease A and the successful air oxidation of the four disulfide bonds has remained a milestone in peptide synthesis (Gutte and Merrifield, 1969,

1971). Synthesis of peptides containing cysteine residues are notorious for causing problems due to the ability of cysteine to form incorrect disulfide bonds. In cases such as with conotoxin G1 where multiple cysteine residues are present, differentially labile sulfhydryl protecting groups have been used to direct formation of the two disulfide bonds (Atherton et al., 1985). Synthesis of rat transforming growth factor I (TGF-I) (Tam et al., 1984) and transforming growth factor ∝ (TGF∝) (Tam, 1987), each of which is a 50-residue peptide, required the oxidation of three disulfide bonds. In both cases, this was accomplished using a mixed disulfide process with reduced and oxidized glutathione (Ahmed et al., 1975).

Sea anemone neurotoxins are similar in size to the TGF∝ and TGF-I polypeptides with between 47 and 49 residues. The tertiary structure of anemone toxins is also stabilized by three disulfide bonds (Rathmayer, 1979). Early attempts to synthesize the anemone toxin Ax I resulted in a peptide which possessed only 11% activity of that of the native toxin (Matsueda and Norton, 1982).

Recently, we purified and sequenced a new neurotoxin from the sea anemone *Stichodactyla helianthus* (Kem et al., 1986). This 48-amino acid peptide has been shown to cause paralysis in both crustacean and mammalian species. However, the toxin has selective toxicity in that it is much more active on crustaceans than on mammals. Investigation of the selective toxicity is a primary goal to aid in understanding this unique structure-

activity relationship. We plan to investigate the importance of particular amino acid residues in this polypeptide toxin through chemical synthesis of mono-substituted analogs. Prior to the synthesis of these analogs, however, it was necessary to synthesize the native toxin sequence in order to determine whether it could adopt the native conformation.

The chemical synthesis of ShN I is described in this chapter. In this way, we hope to be able synthesize substantial amounts of analog toxins which can then be made available for biochemical studies. We characterized the refolded synthetic polypeptide by a variety of chemical and physical methods including: ^{1}H NMR, Edman sequence analysis, and peptide mapping. Finally, we demonstrate that the biological activity of the synthetic toxin is identical to the native toxin on crustaceans, mammals, and crab axolemma vesicles. This is the first time that a polypeptide toxin of this size has been chemically synthesized which possesses biological activity comparable to that of the native toxin.

Experimental Procedures

Materials. Sea anemone toxin I from *Stichodactyla helianthus* was purified as previously described (Kem et al., submitted). [^{3}H]-STX (specific activity 10 Ci/mmol) was kindly provided by Dr. Gary Strichartz (Department of Anesthesia, Harvard Medical School, Boston Massachusetts). $Na^{125}I$ was purchased from New England Nuclear (Boston, Massachusetts) and had a specific

activity of 2200 Ci/mmol. Inc. Both glutathiones (reduced and oxidized) and subtilisin (type VII) were obtained from Sigma Chemical Company (St. Louis, Missouri). Sequenal grade guanidinium HCl was from Pierce Chemical Company (Rockford, Illinois). TTX was obtained from Calbiochem (La Jolla, California). All other reagents were the highest commercial grade available.

Synthesis of ShN I. All t-butyloxycarbonyl amino acids were of the L-configuration except for t-butyloxycarbonyl-Gly and were purchased from Bachem Inc., Torrance, California. The t-butyloxycarbonyl amino acids included: Asp (Benzyl), Arg (Tosyl),Cys (S-methylbenzyl), Glu (Benzyl), Lys (2-chlorobenzyloxycarbonyl), Ser (O-benzyl), Thr (O-benzyl), Trp (Formyl), and Tyr (2-bromobenzyloxy-carbonyl). Asparagine was incorporated into the peptide with an unprotected side chain. Stepwise synthesis proceeded from the C-terminal Lys residue which was anchored with phenylacetamidomethyl linker (Mitchell et al., 1978) to the resin (0.59 mmol/g resin, Applied Biosystems Inc., Foster City, California). Automated assembly of the peptide was performed on the Applied Biosystems 430-A peptide synthesizer using previously reported protocols (Kent et al., 1984) and 2 mmol of symmetric anhydride per mmol of resin. A solution of TFA (60% in dichloromethane) was used for deblocking; diisopropylethylamine (50% in dimethylformamide) was used for neutralization of the resulting ammonium group; resin

washing was accomplished with dichloromethane or methanol. Amino acid residues were activated to the symmetric anhydrides with 0.5 eq of dicyclohexylcarbodiimide in dichloromethane. Couplings (30 min) of the activated amino acids to the peptide chain were carried out in dimethylformamide or mixtures of dichloromethane and dimethylformamide, depending on the solubility of the residue. Recouplings were automatically performed at all β-branched amino acid residues and at those residues containing large bulky aromatic protecting groups. Asn and Arg were each double coupled in the presence of 2 eq of 1-hydroxybenzotriazole. The average coupling efficiency for the entire synthesis was 99.1% monitored by the quantitative ninhydrin method (Sarin et al., 1981). The peptide was simultaneously cleaved from the resin, and deprotected using the low followed by high concentration of HF procedure (Tam et al., 1983). The peptide resin (0.35 g portion) was treated with 2.5 ml of anhydrous HF in the presence of 6.5 ml dimethylsulfide, 0.75 ml p-cresol, and 0.25 ml p-thiocresol for 2 h at 0 °C. At this point, the HF and dimethylsulfide were removed in vacuo and the reaction vessel was recharged with 9.5 ml of anhydrous HF and allowed to react for 1 h at 0 °C. After removal of the HF under reduced pressure, the organic byproducts were extracted with anhydrous diethylether containing 1% β-mercaptoethanol. The free peptide was extracted into 10% acetic acid. The peptide solution was then lyophilized and stored dessicated at -20 °C.

Amino Acid Analysis. For resin samples, an aliquot of peptidyl resin (1-2 mg) was placed into a pyrex tube to which 500 μl of ultrapure 12N HCl and 500 μl propionic acid were added. The tubes were then sealed *in vacuo* and incubated at 110 °C for 24 h. The hydrolysates were dried down, filtered, and analyzed on a Beckman 6300 amino acid analyzer. Peptide samples (100 μg) were hydrolyzed in ultrapure 6 N HCl for 24 h at 110 °C in sealed evacuated tubes. The hydrolysates were then processed as described above.

Folding and Disulfide Bond Formation. The crude peptide (15 mg) was dissolved in 3 ml of 0.1 M Tris HCl, pH 8.5, containing 6 M guanidinium HCl, 1 mM Na_2EDTA. Reduction of the peptide was initiated with addition of 0.2 ml of β-mercaptoethanol to the peptide solution. Following reduction (4 h, 25 °C), the peptide was desalted on a Sephadex G-15 column equilibrated with 10% acetic acid. The reduced peptide was concentrated by lyophilization and redissolved to a concentration of 50 μg/ml in freshly prepared reoxidation buffer consisting of 0.1 M Tris HCl, pH 8.2, containing 10 mM glutathione (reduced) and 1mM glutathione (oxidized) at 25 °C (Ahmed et al., 1975). After 16 h, the peptide solution was concentrated and desalted on a Sephadex G-15 column equilibrated in 10% acetic acid. The peptide fraction was pooled and lyophilized.

Purification. The reoxidized peptide mixture was loaded on a phosphocellulose (Whatman) column equilibrated with 20 mM ammonium formate, pH 4.0. The phosphocellulose column was developed with a linear gradient 20 mM to 500 mM ammonium formate, pH 4.0 (Figure 3:1). The correctly folded neurotoxin (Figure 3:1, peak VI) eluted near the end of this gradient and constituted 22.8% by weight of the starting material. Further purification by reverse phase HPLC on a Waters C-18 μ-bondapak column resolved this peptide to a single homogeneous peak using a gradient of 10% to 50% acetonitrile into 0.1% TFA at a flow rate of 1.5 ml/min. Analysis of the highly purified toxin gave the following ratios with expected values in parentheses: Ala (6) 5.8, Arg (2) 1.95 , Asp (6) 5.61, Glu (2) 1.94, Gly (4) 4.18, Ile (3) 3.00, Leu (2) 2.15, Lys (5) 5.07, Pro (2) 1.94, Ser (2) 1.85, Thr (4) 3.54, Tyr (2) 1.88. Cysteine and tryptophan were destroyed in the analysis and were not included in these values.

Peptide Mapping Experiments. Subtilisin digestions (16 h) of both the synthetic refolded toxin (3.0 nmol) and native toxin (2.75 nmol) were conducted at an enzyme to substrate ratio of 1:100 in 50 mM ammonium acetate pH 6.8 at 25 °C. Analysis of the digests was performed by reverse phase HPLC on a Waters C-18 μ-bonda-pak column using a linear gradient of 10% to 50% acetonitrile into 0.1% TFA in 30 min at a flow rate of 2.0 ml/min monitoring the absorbance change at 254 nm.

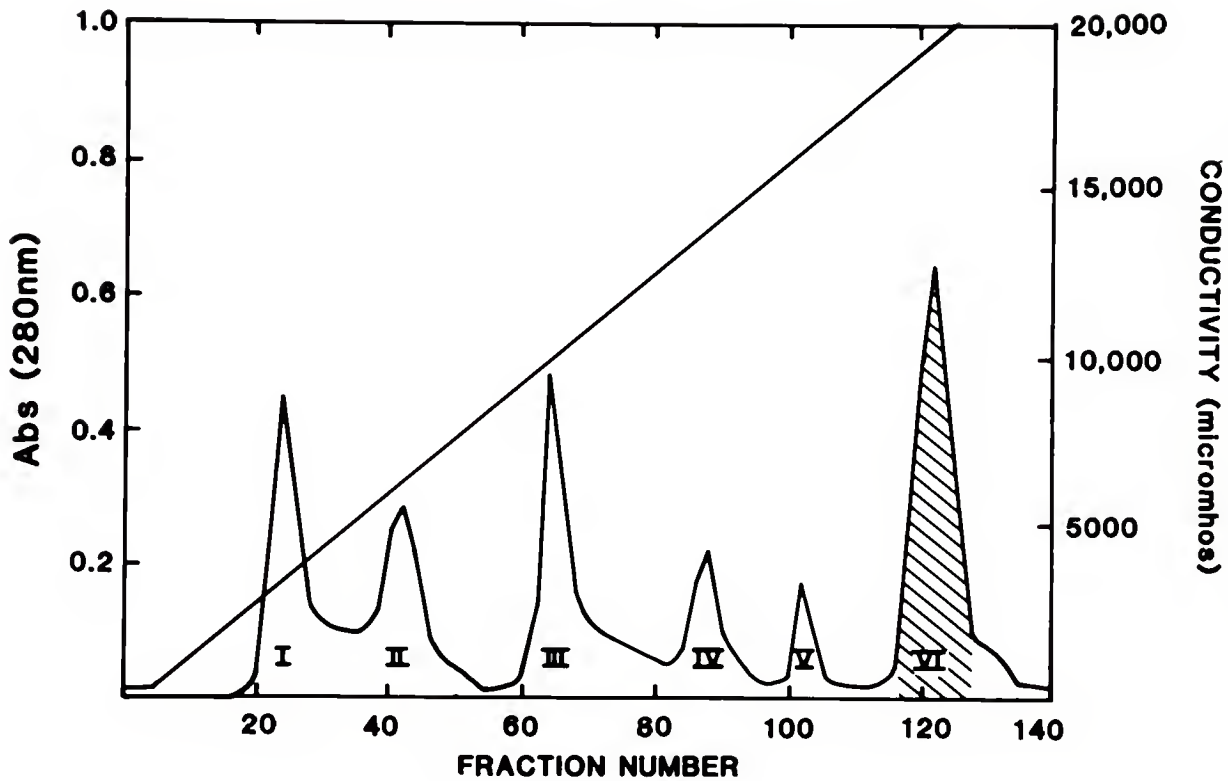


Figure 3:1: Phosphocellulose Ion Exchange Separation of Synthetic ShN I. The crude oxidized synthetic neurotoxin was adsorbed on to a phosphocellulose column equilibrated in 20 mM ammonium formate, pH 4.0. The products were eluted from the column with a linear gradient from 20 mM to 500 mM ammonium formate, pH 4.0. Six peaks were isolated from this column and only peak VI (hatched peak) was shown to have neurotoxic effects.

Sequence Analysis. Approximately 1 nmol of synthetic ShN I (Figure 3:1, peak VI) was loaded on a glass fiber filter derivatized with Polybrene. The sample was then subjected to 10 cycles of degradation in the Applied Biosystems 470 A automated sequenator. The PTH-amino acid residues were then analyzed by reverse-phase HPLC on a Waters Nova-pak C-18 (5 μm particle size) column with a 45 min linear gradient of 10% to 56% methanol into H_2O.

Fluorescence Emission Spectroscopy. Fluorescence emission spectra were obtained using a Perkin Elmer MPF 44-B fluorescence spectrofluorimeter. Synthetic and native toxin were dissolved in 50 mM Na_2HPO_4 and the pH was adjusted with NaH_2PO_4 to 6.8. Spectra were obtained at 25 °C in a 1 cm quartz cell. Aqueous/organic solvent mixtures were performed with ultrapure dioxane (Fluka Chemical Company) and 50 mM Na_2HPO_4, pH 6.8. Scanning speed was 30 nm/min with an excitation wavelength of 290 nm.

Circular Dichroism Spectropolarimetry. Circular dichroism spectra of the synthetic and native toxin were recorded with a Jasco J-500C spectropolarimeter equipped with variable detector geometry. Spectra were obtained with the photomultiplier tube directly adjacent to the cell, which resulted in an acceptance angle of close to 90°. The instrument was calibrated with d(+)-

10-camphorsulfonic acid at 290 and 192.5 nm (Chen and Yang, 1977). Measurements were made with the samples dissolved in 50 mM Na_2HPO_4, pH 6.8, at 25 °C in a calibrated 0.01 cm pathlength cell. Spectra were collected at a scanning speed of 2 nm/min in triplicate to generate an average spectrum from the three scans at a time constant of 16 sec.

NMR Spectroscopy. ^1H NMR spectra were recorded at 300.07 MHz on a Bruker CXP-300 spectrometer operating in a pulsed Fourier transform mode with quadrature detection; 5 mm o.d. spinning sample tubes (Wilmad Glass Co., 527-PP grade) were used. The probe temperature was maintained at 27 °C with a Bruker B-VT 1000 variable temperature unit and calibrated using a methanol standard. Spectral accumulation parameters were: sweep width 3400 Hz, 8192 points, 90° radiofrequency pulses, 2.0 s recycle time, 300-1100 accumulations. Resolution enhancement was effected by means of Lorentzian-Gaussian transformation, and data were zero-filled to 16,384 points prior to Fourier transformation. Chemical shifts were measured digitally, with 1,4-dioxane, at 3.751 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), as internal standard.

Biological Assay. Intrahemocoelic injection of 3-5 g fiddler crabs (*Uca pugilator*) was performed with synthetic or native toxin diluted at a constant interval with saline containing 0.1 mg/ml BSA (142 mM NaCl, 2 mM $CaCl_2$, 40 mM KCl, 9 mM dextrose,

and 10 mM Tris HCl, pH 7.4). Paralytic response was determined 15 min after the injection by placing the animals on their backs and measuring their ability to "right" themselves in a 2 min interval. Intracerebroventricular injection of 24-31 g mice was performed with a constant dose interval of either synthetic or native toxin. At low doses (1-2 μmol/kg), a slight tremor could be detected by holding the animal upside down by its tail. At higher doses, the effects of the toxin resulted in paralysis and eventual death of the animal.

Preparation of Axolemma vesicles. Axolemma membranes were obtained from the Blue Crab (*Callinectes sapidus*), as previously described (Chapter II, this dissertation).

Radiolabeling ShN I with [^{125}I]. The chloramine-T method of iodination (Hunter and Greenwood, 1962) was used to incorporate [^{125}I] into the Tyr residues of ShN I as previously described (Chapter II, this dissertation).

Binding Experiments. [^{125}I]-ShN I and [^{3}H]-STX binding to axolemma vesicles was performed as previously described (Chapter II, this dissertation).

Results

Synthesis of ShN I. The synthetic ShN I, prepared as described under "Experimental Procedures", was characterized by

a number of highly discriminating complementary chemical and analytical techniques in order to test its identity with the natural product. First the fidelity of the chain assembly was assessed by quantitative ninhydrin monitoring of each amino acid coupling (Sarin et al., 1981). An overall coupling efficiency of 99.1% was observed. During the synthesis, several problematic residues were encountered. These residues were located primarily in regions having a high density of hydrophobic residues or residues containing large bulky protecting groups. The Tyr_{35}-Tyr_{36} sequence had the two lowest initial coupling efficiencies observed for the entire synthesis. A second coupling at each of these two residues improved the coupling efficiency to greater than 99%. A double coupling protocol for any suspected problematic region such as those mentioned above significantly reduced the number of deletion sequences in the overall synthesis. Edman sequencing of the synthetic toxin yielded the expected sequence through 10 cycles. No significant "preview" of amino acids was observed, indicating that contamination by deletion peptides was minimal (upper limit of approximately 0.5%). The synthetic toxin was analyzed by reverse-phase HPLC using two different solvent systems (methanol with 50 mM ammonium acetate, pH 4.54 and acetonitrile with 0.1% TFA). The results of HPLC analysis with the TFA/acetonitrile buffer system are shown in Figure 3:2. The synthetic toxin showed a single symmetric peak eluting in the same position as the native toxin (Figure 3:2). In another control experiment, a

1:1 mixture of the synthetic and native toxin was prepared and injected into the same HPLC system. A single sharp peak was observed for this mixture shown in Figure 3:2C. Furthermore, analytical isoelectric focusing of the synthetic toxin showed only one band which migrated with the same pI (8.3) as the native toxin (data not shown).

Physical Characterization. The primary structure of the refolded synthetic ShN I was further analyzed by peptide mapping with the general protease subtilisin (Figure 3:3A). After separation by reverse-phase HPLC, peptides generated by the digestion with subtilisin were identified by amino acid composition. Digestion of the native toxin with subtilisin yielded an identical peptide map as shown in Figure 3:3B. Amino acid composition of both peptide mapping experiments confirmed that the same peptides were generated for both synthetic and native toxins. Attempts to generate peptide maps with more specific enzymes such as trypsin, chymotrypsin, and pepsin were unsuccessful presumably due to the compact structure the toxin possesses when the disulfide bonds are intact.

Both the native and synthetic toxins contain a single tryptophan residue which serves as reporter group for the environment which it experiences. In Figure 3:4 (upper panel), the emission spectra for the native and synthetic toxin are shown. The fluorescence maximum for both samples occurred at 338 nm. When the peptides were dissolved in aqueous/organic

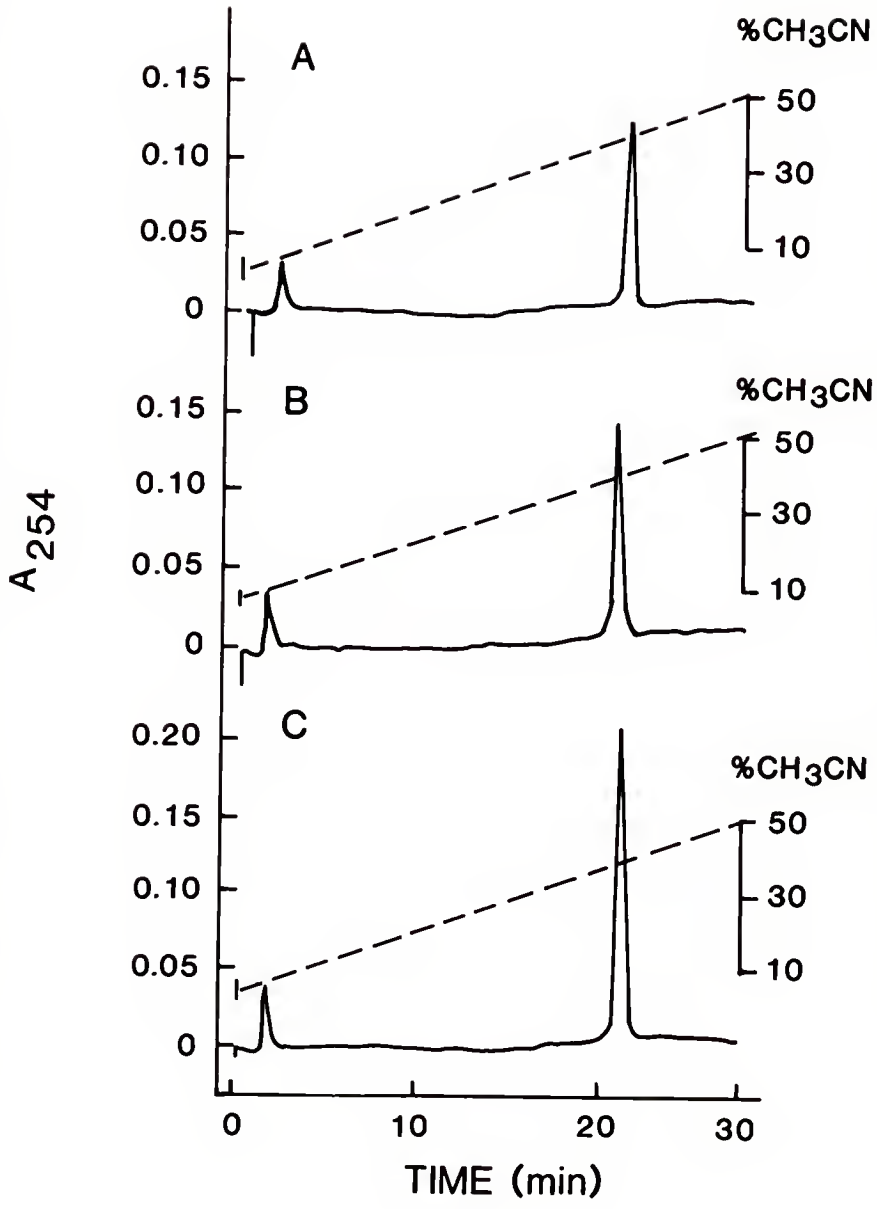


Figure 3:2: Reverse-phase HPLC chromatograms of synthetic and native ShN I. The gradient was linear and consisted of 10 to 50% acetonitrile into 0.1% TFA in 30 min at 1.5 ml/min on a C_{18} column. (A) Injection of 100 μg native ShN I, (B) Injection of 100 μg synthetic ShN I, and (C) coelution of 75 μg of synthetic and 75 μg native ShN I.

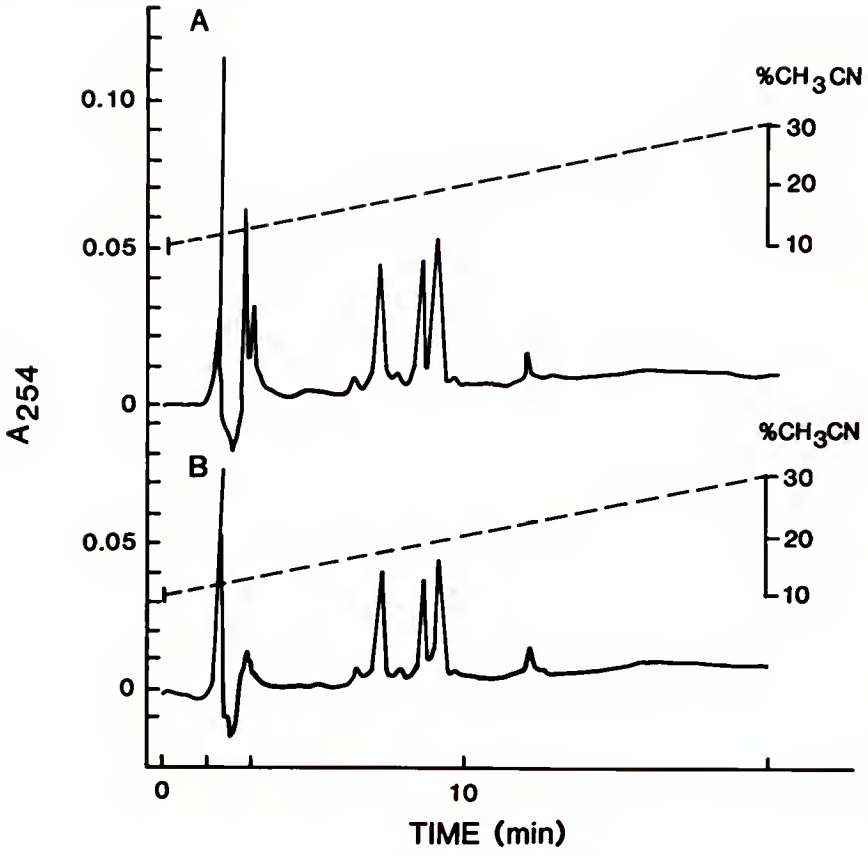


Figure 3:3: Peptide mapping of native and synthetic toxins. Digestion with subtilisin was carried out as described in the text. (A) Synthetic toxin peptide fragment reverse-phase HPLC (C_{18}) elution profile. (B) Native toxin peptide fragment reverse-phase HPLC (C_{18}) elution profile. A linear gradient of 10-30% acetonitrile into 0.1% TFA at a flow rate of 2.0 ml/min was used for the separation.

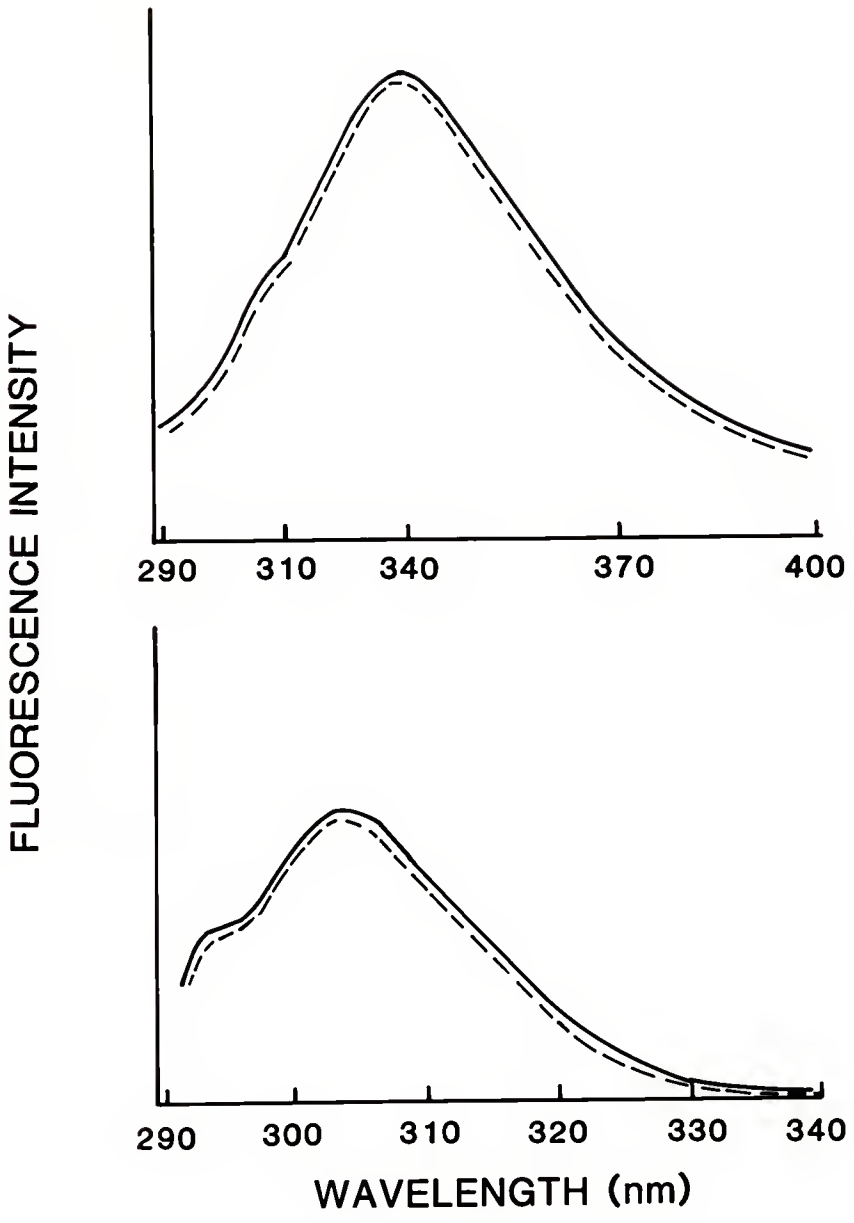


Figure 3:4: Fluorescence emission spectra for synthetic and native ShN I. Excitation wavelength was 290 nm, with the gain set at 10. Native toxin (150 nM) (solid line) and synthetic (150 nM) (dotted line) emission spectra in (Upper panel) 50 mM $NaPO_4$, pH 6.8 at 22 ° C, (Lower panel) 20% dioxane and 80% 50 mM $NaPO_4$, pH 6.8 at 22 °C.

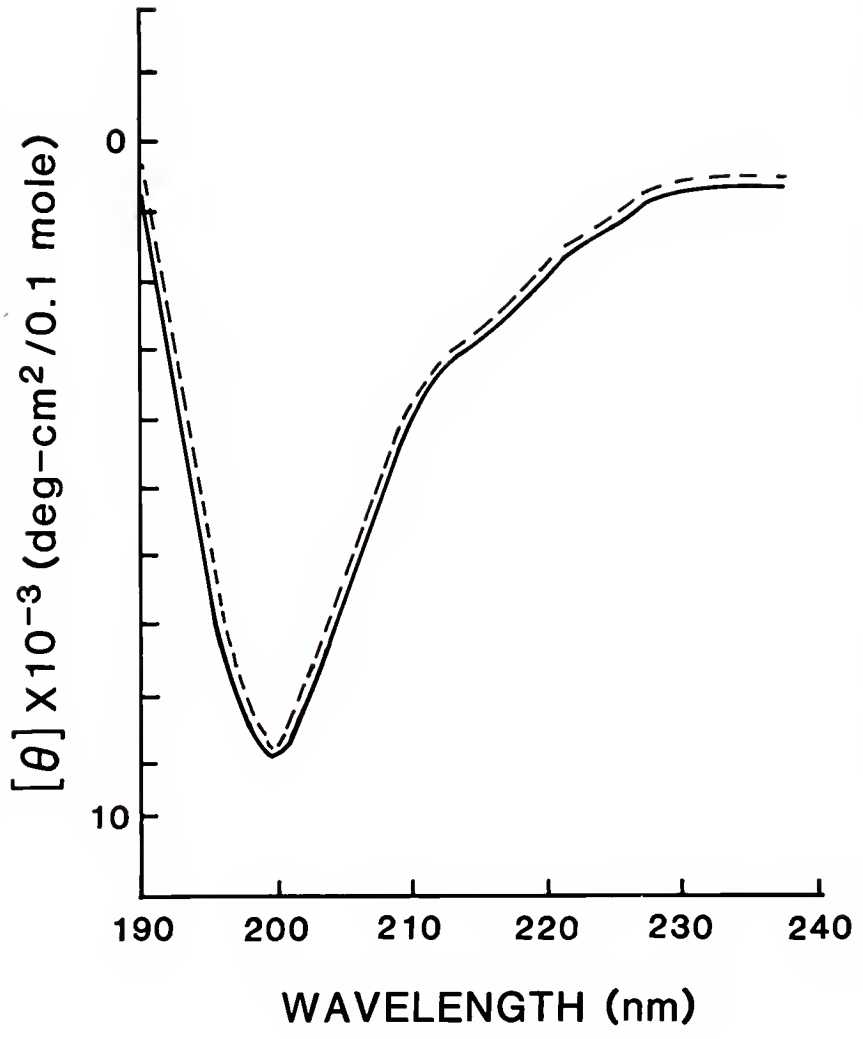


Figure 3:5: Circular dichroism spectra for synthetic and native ShN I. Spectra were obtained as described in text. Each spectrum represents an averaging of three scans for synthetic ShN I (dotted line) and native ShN I (solid line). The concentrations of both synthetic and native toxin were 150 nM.

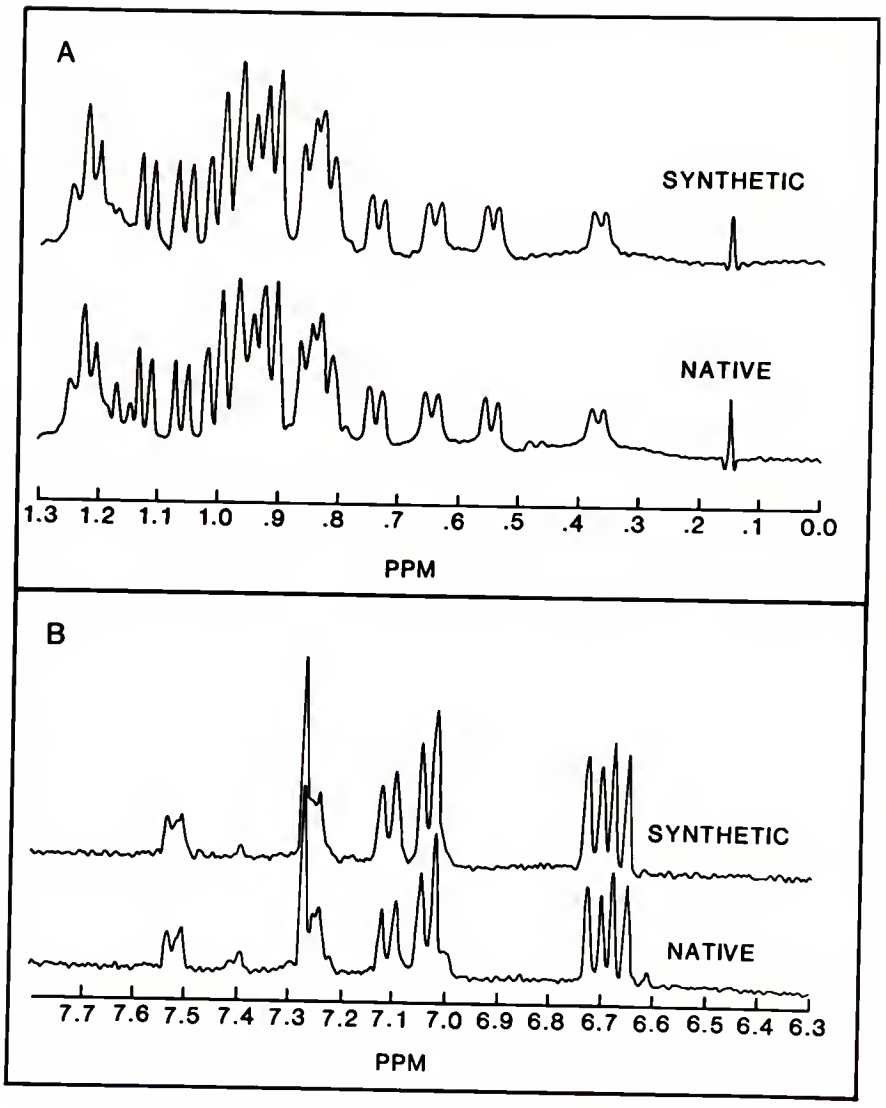


Figure 3:6: Methyl and aromatic regions of 300 MHz ^{1}H NMR spectra of synthetic and native ShN I in $^{2}H_2O$ at 27 °C and pH 5.4. A, Methyl region of 300 MHz spectra. B, Aromatic region of 300 MHz spectra. (Upper trace of each panel) ShN I, 1.9 mM, 300 scans. (Lower trace of each panel) Native ShN I, 0.9 mM, 1060 scans.

cosolvent mixture (dioxane/water), the emission maxima were blue-shifted to a maximum at 304 nm compared to the emission maxima in a purely aqueous system (Figure 3:4 lower panel). From the emission spectra for the tryptophan residue in both solvent systems, it appears to be exposed to the aqueous environment.

Further physical characterization involved analysis of secondary structural elements present of both the synthetic and native toxin. The CD spectra for both of the peptides are shown in Figure 3:5. Both toxin molecules showed a strong negative ellipicity at 198 nm. Also, the shoulder in the 215-225 nm region was identical, indicating the same β-strand region was present.

The high resolution ^{1}H NMR spectra for each of these molecules have been collected. The spectra of the synthetic and native toxin are shown in Figure 3:6. Figure 3:6A shows the methyl region of the spectra. The spectra showed nearly identical splitting patterns further confirming the close similarity of the structure of the synthetic peptide to the native toxin. Fig 3:6B shows the aromatic region of the spectra. Identical splitting patterns were also observed in this region. Also seen in the native toxin spectrum in Figure 3:6B are several peaks at 6.62 ppm and 7.41 ppm which are not observed in the synthetic toxin. These peaks resulted from impurities in the native toxin preparation which were absent from the synthetic toxin. The remainder of each spectrum was

also identical, but involved much spectral overlapping, so it has not been included. A complete assignment of the ^1H NMR spectrum of native ShN I will be published shortly (Fogh et al., in press). For the polypeptides to exhibit this behavior, the tertiary structures are likely to be identical.

Biological Characteristics. Biological activity was conveniently assayed by determining the LD_{50} for the synthetic toxin on fiddler crabs and white mice as described under "Experimental Procedures". These data are presented in Table 3:1. The LD_{50} for the synthetic toxin exhibits the same preferential toxicity for crabs versus mice as the native toxin. To observe any paralytic response, it was necessary to use the intracerebroventricular injection into the brain as opposed to intraperitoneal injection. Intraperitoneal injection had been previously attempted for the native toxin and resulted in almost no effect up to concentrations of 15 mg/kg (Kem et al., submitted). For both synthetic and native toxins, the crabs assumed a flexed posture where the legs were rigidly held bent back into the body. Following intracerebroventricular injection into the mice, a violent tremor onset at approximately 2 min, followed by convulsions. A sudden burst of frantic energy preceded the death of the animal at approximately 4 min.

Displacement of [^{125}I]-ShN I from axolemma vesicles. After thorough characterization of the membrane vesicles, the

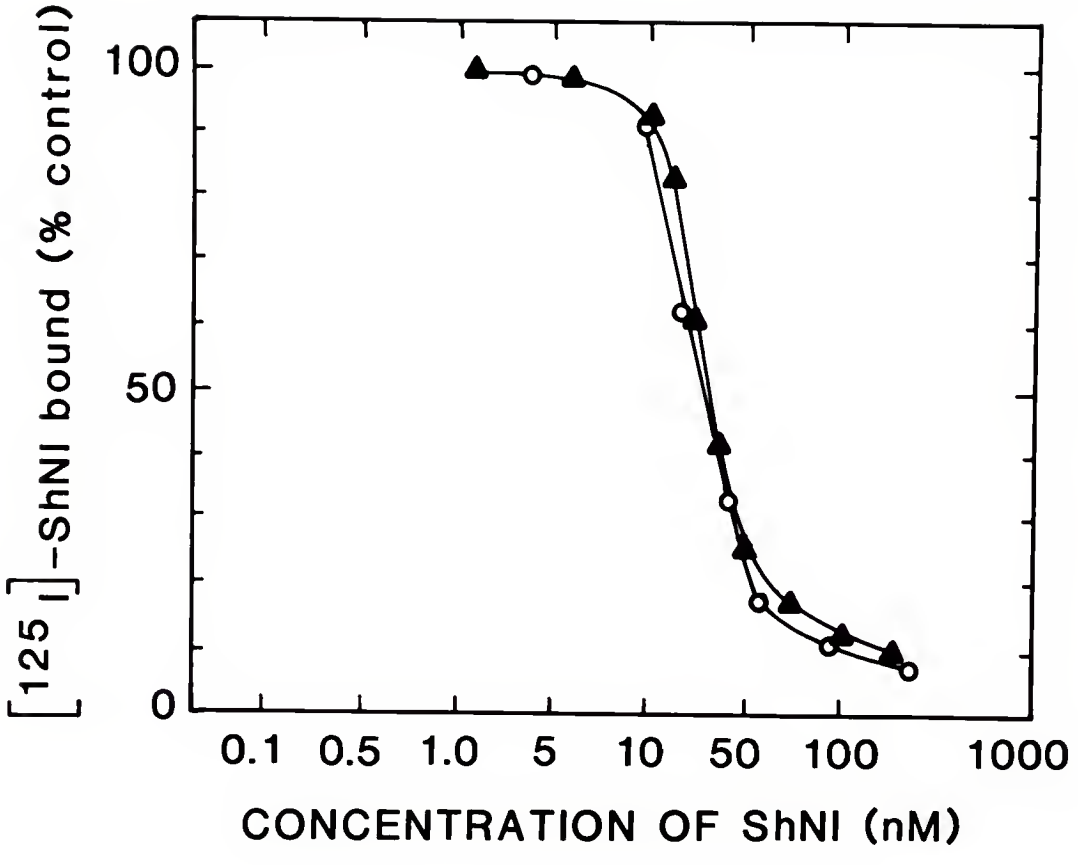


Figure 3:7: Inhibition of specific [^{125}]-ShN I binding by unlabeled native or synthetic ShN I to crab axolemma vesicles. Binding was measured under equilibrium conditions in the presence of the indicated concentrations of (O) ShN I or (▲) synthetic ShN I as described in the text. Nonspecific binding in the presence of 10 μM ShN I was subtracted out.

competitive binding of synthetic toxin versus the [^{125}I]-ShN-I native toxin derivative was measured. This iodinated derivative possesses more than 90% of the toxicity of the native to (Chapter II). Results presented in Figure 3:7 show that the unlabeled synthetic ShN-I (▲) displaces [^{125}I]-ShN-I from its association with axolemma vesicles. The concentration of synthetic ShN that induces half displacement of [^{125}I]-ShN I is $K_{0.5}$ = 28 nM. In a separate experiment the $K_{0.5}$ of the native ShN-I (O) was determined to be 25 nM. Direct titration of this receptor with [^{125}I]-ShN I determined that a single class of binding sites was present with a K_D of 14 nM. Thus, the synthetic and native toxin exhibited nearly identical dissociation constants.

Discussion

The sea anemone *Stichodactyla helianthus* possesses a 48 residue neurotoxic polypeptide which presumably binds to the sodium channel site III receptor. This paper shows that it is possible to chemically synthesize this polypeptide neurotoxin of the sea anemone. During the initial synthesis of the toxin, we observed low coupling yields in the regions of the structure characterized by a high density of hydrophobic residues or at positions such as the aromatic tyrosine residues which have bulky 2-bromobenzyl protecting groups. The Tyr-Tyr sequence at position 36 and 37 represented the lowest initial coupling efficiency observed through out the entire synthesis. This was

most likely due to the steric hinderance imposed by the large bromobenzyl protecting groups on the phenolic hydroxyl group of these two residues. Incorporating a second coupling step at these positions increased the coupling efficiency up to or greater than 99.1% and, thus all future synthesis will use a mandatory double coupling cycle at every residue to maximize the purity of the final product. The automated chemical synthesis of the sea anemone toxin Anthopleurin A (Ax I) has been reported (Matsueda and Norton, 1982). The native toxin is a very potent vertebrate neurotoxin which has also been shown to possess cardiac stimulant properties (Norton et al., 1976). Following reoxidation of the disulfide bonds, Matsueda and Norton (1982) were only able to purify an activity which was approximately 11% of the native toxin. Although no rationale for this low activity was presented, it may have resulted from either cumulative synthetic error or possibly incorrect disulfide pairing.

The reduced native toxin and the crude (reduced) synthetic toxin are essentially devoid of toxic properties (results not shown). Refolding the synthetic neurotoxin by reoxidizing the three disulfide bonds was easily monitored following phosphocellulose ion exchange chromatography by the appearance of a biologically active peak. The refolded purified synthetic polypeptide constituted approximately 20% of the crude synthetic toxin by weight. Native toxin reoxidation was approximately 50%. The lower yield for the synthetic toxin probably results

from the presence of deletion sequences in the crude peptide weight which may be unable to adopt the native conformation. Thus, the oxidative folding process can provide an important purification step which minimizes the problems associated with a peptide containing three disulfide bonds (15 different pairings in the monomeric form).

The synthetic toxin physical structure was compared to the native toxin using: fluorescence emission, circular dichroism, and ^{1}H NMR. The spectra obtained from all of these different techniques strongly suggest that the synthetic toxin structure was identical to the native toxin. Peptide mapping experiments with the proteases subtilisin and thermolysin (unpublished results) were used to generate peptide maps of both synthetic and native toxin . The identity of these peptide maps indicate that the same susceptible groups are present in both synthetic and native toxins. Both synthetic and native toxins were refractory to cleavage by the more specific proteases such as trypsin and pepsin (results not shown). These enzymes failed to generate peptide fragments probably because the tightness of the structure caused by formation of the disulfide bonds prevented binding in the active site of these proteases.

One of the most interesting properties of ShN I is the species selectivity which the native toxin exhibits. The synthetic toxin showed identical LD_{50} when compared with the native toxin with respect to toxicity for crustacean and mammalian species (Table 3:1). Analysis of toxin binding

Table 3:1. Pharmacological Properties of Synthetic ShN I

	Toxicity		Axolemma[c]
	Crab LD_{50}[a] ($\mu g/kg$)	Mice LD_{50}[b]	Vesicle Binding K_D (nM)
Native ShN I	2.92	116.0	25
Synthetic ShN I	3.14	115.6	28

[a]Injected into the intrahemocoelic space.

[b]Injected into intracisternal space.

[c]Binding to axolemma vesicles prepared from Blue Crab walking leg nerve.

properties on the crab preparation which has been studied for the native toxin with [^{125}I]ShN I derivative (Chapter II, this dissertation), showed the synthetic toxin had an approximately identical K_D value (28 nM) as the native toxin (25 nM) (Table 3:1, Figure 3:7).

Several different groups of sea anemone polypeptide toxins have been isolated. The first group consists of polypeptides which are termed the short toxins such as *Anemonia sulcata* toxin III (Beress et al., 1975, 1977) and *Parasicyonis actinostoloides* toxin (Nishida et al., 1983) which contain 27-31 amino acid residues. The second group consists of the long neurotoxins which contain 46-48 amino acid residues. These long toxins have been recently divided into two classes based upon sequence homology (Schweitz et al., 1985; Wemmer et al., 1986; Kem et al., submitted). Members of the each class possess approximately 60% homology, however, there is only about 30% homology between the two classes. The majority of this homology between classes resides in the positioning of the cysteine residues which are identical for all of the toxins isolated. Interestingly, each of these long toxin classes come from different sea anemone taxonomic family. The first anemone toxins, As II and *Anthopleurin* A (Ax I), that were isolated and sequenced belonged to the family Actiniidae (Wunderer et al., 1976; Tanaka et al., 1977). These toxins are two members of the group that have been designated the type 1 long toxins. More recently, several toxins from the family Stichodactylidae have

been isolated (Zykova et al., 1985; Wemmer et al., 1986; Metrione et al., 1987; Kem et al., submitted). These have been designated the type 2 long anemone toxins.

These two classes are immunologically distinct as might be expected from the dissimilarities in the amino acid sequences (Schweitz et al., 1985; Chapter II, this dissertation). However, it is not yet clear whether these toxins bind to the same or to separate receptors. Data for Hp II (formerly *Radianthus paumotensis*) has shown that it is unable to displace [^{125}I]-As V from rat brain synaptosomes (Schweitz et al., 1985). Hp II does not displace the iodinated scorpion toxin derivative [^{125}I]-AaH II, another toxin specific for site III on the sodium channel, from the same synaptosome preparation (Schweitz et. al., 1985). More recently, we obtained data that confirmed these results for the binding of [^{125}I]-ShN I to Blue Crab walking leg axolemma vesicles. We have shown that As II was unable to displace [^{125}I]-ShN I from this preparation. Similarly, using [^{125}I]-AaH II (scorpion site III toxin) on rat brain synaptosomes we were able displace the [^{125}I]-AaH II with As II but concentrations of ShN I up to 50 μM failed to displace the iodinated scorpion toxin (Pennington et al., Chapter II, this dissertation). Thus, the possibility exists for preferential binding of anemone toxins to different parts of the site III receptor or a different receptor site altogether.

The class II long toxins have the widest distribution of toxicity with respect to crustaceans and mammals. Comparing the

sequence differences between these toxins reveals that the two toxins with the greatest differential toxicity (ShN I and Hm II) possess nearly 80% sequence identity. Among those residues that are not identical are conservative changes such as Ile for Leu or Ser for Thr. Thus, only six nonconservative amino acid substitutions exist between the two toxins yet they exhibit the two extremes in species specific toxicity. Now that synthesis of a type 2 sea anemone toxin has been shown to be feasible, we should be able to determine which amino acid residues are important for sodium channel binding in either mammalian and crustaceans, thereby we may begin to understand the different phylogenetic toxicities of certain sea anemone polypeptides.

CHAPTER IV

STRUCTURE-FUNCTION STUDIES ON THE MAJOR NEUROTOXIN OF *STICHODACTYLA HELIANTHUS*: IDENTIFICATION OF ESSENTIAL RESIDUES USING SYNTHETIC MONOSUBSTITUTED ANALOGS

Introduction

The acidic and basic residues of proteins are the most frequently exposed residues on the surface of proteins (Provencher and Glockner, 1981). While the hydrophobic effect of removing ordered water molecules from associating protein-protein interfaces is the major thermodynamic force stabilizing such complexes, the complementarity of the two surfaces plays a selective role in determining which proteins may associate (Chang et al., 1978). Both nonpolar as well as polar residues determine the complementarity between the two proteins or ligand and receptor. In the case of the long sea anemone polypeptide neurotoxins, a surface hydrophobic patch containing the aromatic residues Trp_{23} and Tyr_{25} has been identified on As I and Ax II (Gooley et al., 1986). Furthermore, these polypeptides all have polarized structures with acidic residues at the N-terminus and basic residues at the C-terminus.

Chemical modification has been used to investigate structure-function relationships in sea anemone toxins. Modification reactions where the carboxyl groups are activated with a water soluble diimide followed by treatment with a nucleophile such as

glycine ethyl ester have been performed on As II and Ax I (Barhanin et al., 1981; Kolkenbrock et al., 1983; Gruen and Norton, 1985). The results of these experiments are difficult to interpret due to the conflicting reports presented. In one report, carboxyl modification destroys the neurotoxicity of As II but does not alter the binding properties (Barhanin et al., 1981). Kolkenbrock et al. (1983) was unable to corroborate these results. Gruen and Norton (1985) observed loss of activity following carboxyl modification of Ax I at position 7 and/or 9.

El Ayeb et al. (1986) were able to assign the epitope which a monospecific antibody recognized on As II using this method of carboxyl modification. Using this antibody, they suggested that the residues of Asp_7 and/or Asp_9 and the C-terminal carboxylate of Gln_{47} were not involved in the receptor binding domain.

Furthermore, from NMR studies, two abnormally low pK_a values of 2 and 3.1 have been reported for Asp carboxylate residues located at positions 7 and 9 in As II and Ax I (Norton and Norton, 1979; Norton et al., 1980). In the case of As I, only the pK_a value of 3.1 was observed by ^1H NMR due to the substitution of Lys at position 7 in the sequence (Gooley et al., 1984). The low pK_a is believed to result from the formation of a salt bridge with Lys_{37} in all of these molecules.

Modification of the basic residues has also been reported by several groups (Vincent et al., 1980; Barhanin et al., 1981; Kolkenbrock et al., 1983). Once again, conflicting data keeps

one from assigning essential residues. The importance of the Arg_{14} (Arg_{13} in type 2 anemone toxins) is implied by its absolute conservation in all long anemone sequences. El Ayeb et al. (1986) showed that residues Arg_{14}, Lys_{35}, and Lys_{36} are not accessible to antibody when bound to the receptor. These results suggested that these residues may be involved in the receptor binding domain.

Recently, a second type of long sea anemone neurotoxin was isolated from anemones belonging to the Stichodactylid family (Schweitz et al., 1981; Kozlovoskaya et al., 1982 Schweitz et al., 1985; Kem et al., submitted). These toxins display approximately 30% sequence identity with the anemone toxins isolated from Actiniid family (type 1 anemone toxins). Other than the identical positioning of the cysteine residues, these molecules have the largest number of homologous residues in common with the type 1 anemone toxins in the N-terminal 13 residues. Among those in common are acidic residues at relative positions of 7 and 9 as well as Arg_{13}. All of the type 2 anemone toxins have lysine at position 4, a tri-anionic stretch from residue 6 through 8, a tryptophan at position 30, and a tetra-cationic cluster at the C-terminus.

Both of these types of anemone toxins appear to elicit similar effects. These type 2 anemone toxins also delay inactivation of the Na channel like the type 1 anemone toxins (Sorokina et al., 1984; Schweitz et al., 1985; Chapter 2, this dissertation). However, these toxins are unable to

competitively displace type 1 anemone toxins in in vitro binding studies, and Hp II and ShN I were unable to displace radiolabeled α-scorpion toxins in these in vitro binding studies (Schweitz et al., 1985; Chapter 2, this dissertation). These results suggested a new receptor site for these type 2 anemone toxins.

Characterization of this receptor with ShN I has determined it not to be affected by membrane potential or have enhanced binding in the presence of veratridine. The receptor was detected in binding studies in both crab axolemma vesicles and rat brain synaptosomes (Chapter II, this dissertation). Furthermore, Schweitz et al. (1985) had previously determined that Hp toxins did not affect β-scorpion toxin binding.

Having identified a new receptor site for the type 2 anemone toxins, we wanted to examine the role of the high degree of charge localization in the N-terminal region of the polypeptide. The major problem with attempting this by chemical modification would be the lack of specificity which the chemical reagents have, as well as the subsequent isolation and determination of the site of modification. Assembly of peptide analogs by solid-phase methods would allow for the incorporation of a desired substitution at an exact position within the polypeptide chain. Thus, eliminating the problems associated with the chemical modification approach.

We continue our studies on the characterization of this receptor with the synthetic approach that we developed for the

total synthesis of the native ShN I molecule (Pennington et al., 1987; Chapter III, this dissertation). In this chapter, we describe the synthesis of six analogs all directed at determining the role of the clustering of ionic charges in the N-terminal region of the ShN I molecule. We have characterized each of these refolded analog neurotoxin molecules by CD, fluorescence emission, and HPLC. The pharmacological properties of each of these analogs were determined in both in vivo and in vitro systems which we have developed previously.

Experimental Procedures

Materials. Sea anemone toxin I from *Stichodactyla helianthus* was purified as described previously (Kem et al., submitted). [^{3}H]-STX (specific activity 10 Ci/mmol) was kindly provided by Dr. Gary Strichartz (Department of Anesthesia, Harvard Medical School, Boston Massachusetts). [^{3}H]-Iodoacetic acid and Na^{125}I were purchased from New England Nuclear (Boston, Massachusetts) and had specific activities of 200 mCi/mmol and 2200 Ci/mmol, respectively. Both glutathiones (reduced and oxidized) were obtained from Sigma Chemical Company (St. Louis, Missouri). Sequenal grade guanidinium HCl was from Pierce Chemical Company (Rockford, Illinois). Thioredoxin and TTX were obtained from Calbiochem (La Jolla, California). All other reagents were the highest commercial grade available.

Synthesis of Analog ShN I peptides. All t-butyloxycarbonyl amino acids were of the L-configuration except for t-butyloxycarbonyl-Gly and were purchased from Bachem Inc., Torrance, California. The t-butyloxycarbonyl amino acids included: Asp (Benzyl), Arg (Tosyl),Cys (S-methylbenzyl), Glu (Benzyl), Lys (2-chlorobenzyloxycarbonyl), Lys N∈-acetyl, Ser (O-benzyl), Thr (O-benzyl), Trp (Formyl), and Tyr (2-bromobenzyloxy-carbonyl). Asn and Gln were incorporated into the peptide with an unprotected side chain. Stepwise synthesis proceeded from the C-terminal Lys residue which was anchored with phenylacetamidomethyl linker (Mitchell et al., 1978) to the resin (0.59 mmol/g resin, Applied Biosystems Inc., Foster City, California). Automated assembly of the peptide was performed on the Applied Biosystems 430-A peptide synthesizer using previously reported protocols (Kent et al., 1984) and 2 mmol of symmetric anhydride per mmol of resin. A solution of TFA (60% in dichloromethane) was used for deblocking; diisopropyl-ethylamine (50% in dimethylformamide) was used for neutralization of the resulting ammonium group; resin washing was accomplished with dichloromethane or methanol. Amino acid residues were activated to the symmetric anhydrides with 0.5 eq of dicyclohexylcarbodiimide in dichloromethane. Couplings (30 min) of the activated amino acids to the peptide chain were carried out in dimethylformamide or mixtures of dichloromethane and dimethylformamide depending on the solubility of the residue. Double coupling of every amino acid residue was

automatically performed. Asn, Arg, and Gln were each double coupled in the presence of 2 eq of 1-hydroxybenzotriazole. Synthesis of each of the analog peptides was accomplished by proceeding with the solid phase assembly of the ShN I sequence until the double coupling of the residue which was immediately before the substitution site. At this time, the resin was washed with methanol and dried with N_2 gas. The resin was then weighed and an aliquot was removed. Synthesis was resumed on the remaining resin until the next substitution site was encountered and the procedure mentioned above was performed. After the removal of resin aliquots at all the desired analog positions, assembly of the native toxin was completed as a control. The synthesis of the monosubstituted analogs was then accomplished by replacing one of the resin aliquots back into the reaction vessel at which time the substituted amino acid residue was coupled followed by coupling of the remainder of the sequence. Coupling of t-Boc-Lys Nϵ-acetyl for the N-acetyl Lys_4 analog was performed manually by dissolving the derivative in DMF and transferring this to the activator vessel. This derivative was then coupled to the peptide resin using a 2 to 1 excess of 1-hydroxybenzotriazole. Three couplings of this derivative were performed and quantitative ninhydrin analysis (Sarin et al., 1981) of the resin indicated greater than 99.9% coupling efficiency. From 1.0 mmol of resin, analogs at six different positions in the N-terminal region were synthesized. For each of analog, the peptide was simultaneously cleaved from

the resin and deprotected using the low followed by high concentration of HF procedure (Tam et al., 1983). The peptide resin (0.35 g portion) was treated with 2.5 ml of anhydrous HF in the presence of 6.5 ml dimethylsulfide, 0.75 ml p-cresol, and 0.25 ml p-thiocresol for 2 h at 0 °C. At this point, the HF and dimethylsulfide were removed in vacuo and the reaction vessel was recharged with 9.5 ml of anhydrous HF and allowed to react for 1 h at 0 °C. After removal of the HF under reduced pressure, the organic byproducts were extracted with anhydrous diethylether containing 1% β-mercaptoethanol. The free peptide was extracted into 10% acetic acid. The peptide solution was then lyophilized and stored dessicated at -20 °C.

Folding, Disulfide Formation, and Purification. The crude peptide (15 mg) was dissolved in 3 ml of 0.1 M Tris HCl, pH 8.5, containing 6 M guanidinium HCl, 1 mM Na_2EDTA. Reduction of the peptide was initiated with addition of 0.2 ml of β-mercapto-ethanol to the peptide solution. Following reduction (4 h, 25 °C), the peptide was desalted on a Sephadex G-15 column equilibrated with 10% acetic acid. The reduced peptide was then diluted with dH_2O (approximately 225 ml) to a concentration of 50 μg/ml as determined by the A_{280}. Analysis of the total sulfhydryl content was by the method of Ellman (1959). The method used for oxidation of the disulfide bonds was based upon the method of Ahmed et al. (1975) using the presence of reduced and oxidized forms of glutathione. The optimal conditions

established for all of the analogs involved the slow dialysis reoxidation method used by Sabatier et al. (1987) for the refolding and reoxidation of AaH II. Following quantitation of the total sulfhydryl content, the diluted reduced peptide solution was place into Spectrapore-6 dialysis tubing (1,000 MW cutoff). The dialysis tubing was then placed into 2 l of freshly prepared reoxidation medium (0.1 M Tris acetate, pH 8.5 containing 0.1 M NaCl, 0.1 M guanidinium HCl, 0.1 mM GSH and GSSG). Gentle stirring of the reoxidation medium was initiated to insure more rapid equilibration of the peptide solution inside the tubing with the refolding buffer. The refolding was allowed to continue for 72 h at 4 °C, over which time the pH of the solution was maintained at 8.5 by the dropwise addition of 1.0 M NaOH. After 36 h, the reoxidation buffer was replaced with 2 l of freshly prepared reoxidation medium. After 72 h, the refolding process was terminated by removing the reoxidation medium and replacing it with 2 l of dH_2O. After 6 h to equilibrate versus the dH_2O, the contents from the dialysis tubing were removed and concentrated by lyophilization. Complete removal of any residual guanidinium or glutathione salts was accomplished by redissolving the lyophilized reoxidized peptide solution into 5 ml of 10% acetic acid and loading this onto a Sephadex G-15 column (1.25 X 100 cm) equilibrated in 10% acetic acid. The reoxidized peptide mixture eluted in the V_o of the column. These fractions were pooled and concentrated by lyophilization.

Thioredoxin Reoxidation. Thioredoxin reoxidation of ShN I analogs was by the method of Pigiet and Schuster (1986). Thioredoxin (ox) was dissolved in 0.1 M Tris HCl, pH 7.4 to a concentration of 100 nM. A sample from this solution was removed and diluted 1:5 into 10 μM dithiothreitol buffered with 0.1 M Tris HCl, pH 7.4 containing 1 mM EDTA, and incubated for 30 min at 25° C. Lyophilized polymeric fractions of Tyr_1 isolated from the V_o of the Sephadex G-75 column were dissolved in the oxidized thioredoxin buffer to a concentration of 100 μM. The reoxidation reaction was initiated when an aliquot of the reduced thioredoxin solution (final concentration 10 μM) was added to the reaction mixture. The extent of monomer formation was assayed by removing samples at 16 h and 48 h and separating them on a Sephadex G-75 column equilibrated in 50 mM ammonium acetate, pH 6.8. The thioredoxin treated monomer was then assayed for neurotoxic activity as described below.

Air Reoxidation. The reduced peptide was isolated from the Sephadex G-15 column equilibrated in 10% AcOH. The peptide was directly diluted into 50 mM ammonium acetate, pH 7.5 to a concentration of 50 μM. The pH of the solution was readjusted back to 7.5 with 1N NaOH. Reoxidation was allowed to continue for up to 96 h or until the test for free sulfhydryls was negative (Ellman, 1959).

Purification of Refolded Peptides. A portion of the lyophilized desalted peptide (10 mg) was then dissolved into 500 μl of 50 mM ammonium acetate, pH 6.8. This solution was then loaded onto a Sephadex G-75 column (0.8 X 180 cm) equilibrated in 50 mM ammonium acetate, pH 6.8. This column was then developed at a flow rate of 12 ml/hr while monitoring the absorbance at 280 nm. The fractions containing peptide were assayed for neurotoxic activity on fiddler crabs as described below. Activity was observed to be associated with a peak eluting at approximately the same elution volume as insulin had during a previous calibration run. The fractions which possessed paralytic activity on crabs were pooled and concentrated by lyophilization. The active peak was further purified using the phosphocellulose cation exchange column procedure employed for purification of the synthetic native sequence (Pennington et al., 1987). The activity was generally localized to peaks eluting at slightly higher conductivities than observed for the synthetic native toxin in the case of the carboxamide analogs, and nearly the same conductivities as that of the synthetic native toxin in the case of the replacements of lysine with N-acetyl lysine and alanine with tyrosine. Reverse phase HPLC purification to obtain a homogeneous refolded analog toxin was performed on a Waters C_{18} μ-bonda-pak column. An initial linear gradient of 30% to 40% acetonitrile into 0.1% TFA at a flow rate of 2.0 ml/min was used. Fractions were collected, evaporated, redissolved in dH_2O and assayed for

paralytic activity on fiddler crabs. The active peak was then rechromatographed on reverse phase HPLC using isocratic elution conditions of 33% acetonitrile at a flow rate of 2.0 ml/min. Fractions were collected and assayed as above for activity. A final isocratic reverse phase step with a slightly higher concentration of acetonitrile (35%) was used to obtain a single homogeneous analog peak. The active analogs were always freeze dried and stored at -20 °C until needed.

Amino Acid Analysis. For resin samples, an aliquot of peptidyl resin (1-2 mg) was placed into a pyrex tube to which 500 μl of ultrapure 12N HCl and 500 μl propionic acid were added. The tubes were then sealed in vacuo and incubated at 110 °C for 24 h. The hydrolysates were dried down, filtered and analyzed on a Beckman 6300 amino acid analyzer. Peptide samples (100 μg) were hydrolyzed in ultrapure 6 N HCl for 24 h at 110 °C in sealed evacuated tubes. The hydrolysates were then processed as described above.

Carboxymethylation. A sample of the native toxin (200 μmol) was reduced in 0.1 M Tris HCl, pH 8.2 buffer containing 6 M guanidinium HCl, 1 mM EDTA and a 100 fold molar excess of β-mercaptoethanol for 16 h at 25° C. Carboxymethylation of the sulfhydryl groups was performed in the dark by adding a 1.1 molar excess of Na iodoacetate dissolved in 10% ethanol containing 1 μCi of [^{3}H]-iodoacetic acid to the reaction vile.

The reaction was terminated after 30 min by adding 100 μl of glacial acetic acid. The derivatized material was then dialyzed exhaustively to remove the salts and unreacted material. Carboxy-methylcysteine values were determined by incorporation of tritium as well as amino acid analysis of the hydrolyzed protein.

Fluorescence Emission Spectroscopy. Fluorescence emission spectra were obtained using a Aminco-Bowman spectrofluorimeter. Synthetic analogs and native toxin were dissolved in 200 mM Na_2HPO_4 and the pH was adjusted with NaH_2PO_4 to 6.8. Spectra were obtained at 25 °C in a 1 cm quartz cell. Scanning speed was 30 nm/min with an excitation wavelength of 290 nm.

Circular Dichroism Spectropolarimetry. Circular dichroism spectra of the synthetic and native toxin were recorded with a Jasco J-500C spectropolarimeter equipped with a variable detector geometry. Spectra were obtained with the photomultiplier tube directly adjacent to the cell, which resulted in an acceptance angle of close to 90°. The instrument was calibrated with d(+)-10-camphorsulfonic acid at 290 and 192.5 nm (Chen and Yang, 1977). Measurements were made with the samples dissolved in 50 mM Na_2HPO_4, pH 6.8, at 25 °C in a calibrated 0.01 cm pathlength cell. Spectra were collected at a scanning speed of 2 nm/min in triplicate to generate an average spectrum from the three scans at a time constant of 16 sec.

Biological Assay. Intrahemocoelic injection of 3-5 g fiddler crabs (*Uca pugilator*) was performed with synthetic or native toxin diluted at a constant interval with saline containing 0.1 mg/ml BSA (142 mM NaCl, 2 mM $CaCl_2$, 40 mM KCl, 9 mM dextrose, and 10 mM Tris HCl, pH 7.4). Paralytic response was determined 15 min after the injection by placing the animals on their backs and measuring their ability to "right" themselves in a 2 min interval.

Preparation of Axolemma vesicles. Axolemma membranes were prepared from the Blue Crab (*Callinectes sapidus*) walking leg and chelae axons by the procedure described previously (Chapter II, this dissertation). Characterization of these vesicles with [^{3}H]-STX consistently had values of approximately 15 pmol [^{3}H]-STX bound per mg protein.

Radiolabeling ShN I with [^{125}I]. ShN I was radiolabeled using chloramine-T procedure (Hunter and Greenwood, 1962) for ^{125}I-iodination of tyrosine residues as previously described (Chapter II, this dissertation).

Binding Experiments. [^{125}I]-ShN I and [^{3}H]-STX binding to axolemma vesicles was measured by rapid filtration assay with glass fiber filters (Whatman GF/B). The standard binding medium consisted of 140 mM choline chloride, 50 mM Hepes adjusted to pH

7.4 with Tris base, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml of bovine serum albumin. Samples were mixed and incubated for 30 min at 4 °C for [^{3}H]-STX and 37 °C for [^{125}I]-ShN. Following 30 min incubation, the samples were diluted with 3.0 ml of ice cold wash buffer (163 mM choline chloride, 5 mM Hepes (adjusted to pH 7.4 with Tris base), 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 0.1 % bovine serum albumin) and immediately collected on glass fiber filters and washed with 3 X 3.0 ml of ice cold binding medium. The bound radioactivity was then determined by liquid scintillation counting for tritium or gamma emission for [^{125}I]. Non-specific binding was measured in the presence of excess ligand (150 nM TTX or 10 μM ShN) and subtracted from the results. Binding measurements at each ligand concentration were done at least in duplicate, and the error measured for each point was less than 3%.

Results

Peptide Synthesis. Solid phase assembly of the analog toxins routinely incorporated a double coupling of every amino acid residue. Use of this procedure was based upon the quantitation of the coupling efficiency during the assembly of the native sequence. Although quantitation of the coupling efficiency at each residue for the analogs was not determined, an estimation can be made that the average coupling efficiency is greater than 99.1%. This estimation is based on the previous results obtained for assembly of the native sequence which only used a

double coupling step at the β-branched amino acid residues or those which were protected with bulky aromatic groups (Pennington et al., 1987; Chapter II, this dissertation). Results of amino acid analysis of the fully assembled analog toxin molecules are shown in Table 4:1. Each of the synthetic analog peptides incorporated a substitution at one of the charged residues in the N-terminal 11 residues. The positions of these six substitution are shown in Figure 4:1. As shown, six singly substituted analogs incorporating conservative isosteric substitutions were made to probe the importance of each of these ionic charges.

Reoxidation of Disulfide Bonds. The native toxin structure pairs the six cysteine residues in three intramolecular disulfide bridges. The synthetic scheme for refolding the native toxin established a oxidoreduction system for the formation of these disulfide bonds using reduced and oxidized glutathione (Pennington et al., 1987). Attempting this same reoxidation method directly on the synthetic analogs resulted in the formation of a precipitate within 1 hour. This precipitate was completely resistant to all of the resolubilization techniques tested.

Solubility during the refolding process was determined to be the first problem to be solved. Using the method employed by Sabatier et al. (1987) for the refolding of scorpion toxin

Table 4:1: Amino Acid Analysis of ShN I Analog Peptidyl Resins[a]

	ShN I	Tyr_1	$NacK_4$	Asn_6	Asn_7	Gln_8	Asn_{11}
Asp	6.0	5.71	5.85	6.21	6.23	5.86	5.91
Thr	4.0	3.30	3.53	4.01	4.15	3.41	3.94
Ser	2.0	1.20	1.52	1.47	1.94	1.62	1.82
Glu	2.0	1.41	1.74	2.16	2.32	2.21	2.08
Pro	2.0	1.92	2.46	1.98	2.10	2.46	1.92
Gly	4.0	3.74	4.00	4.28	4.55	4.25	4.45
Ala	6.0	5.00	6.00	6.00	6.00	6.00	6.00
Val	1.0	1.01	1.08	1.10	1.28	1.01	1.04
Ile	3.0	2.57	2.97	3.23	3.17	2.68	2.93
Leu	2.0	1.86	2.11	2.11	2.23	1.81	2.08
Tyr	2.0	2.23	1.88	1.89	1.82	1.67	1.46
Lys	5.0	4.68	5.31	5.09	4.91	5.12	5.21
Arg	2.0	2.15	2.06	2.12	2.27	1.78	2.06

[a]Hydrolysates from 1:1 12N HCl and propionic acid, 110° C, 24 h. Analysis by ninhydrin method. Cys and Trp were not determined. All values based upon Ala.

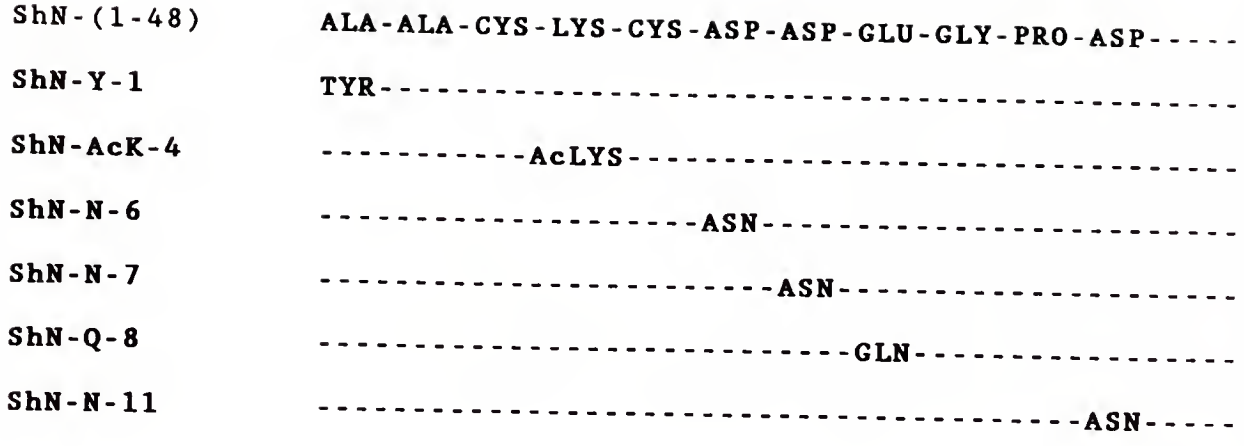


Figure 4:1: Analog Substitution Sites in ShN I. The six monosubstituted analogs used in these studies are diagrammed such that only the site where a substitution was made has been indicated. The remainder of the sequence (47 residues) for each analog is identical.

AaH II, we found this method to give the best results for the recovery of total soluble peptide. A summary of the amounts of soluble peptide are presented in Table 4:2. Only the Tyr_1 and Asn_{11} analogs were observed to form precipitates using this technique, however, the amount of soluble peptide was much greater than any other reoxidative methods that were attempted for these two analogs.

Purification of the Refolded Analogs. The first step in the purification process for the analogs was gel filtration through Sephadex G-75 in 50 mM ammonium acetate, pH 6.8. This column step was successful in separating the refolded peptide mixture into three major components for each of the analogs (Figure 4:2). The first peak was high molecular weight material which was most likely a mixture of different intermolecular disulfide bonded polymers. The second peak generally eluted at a position which would indicate a higher molecular weight than monomeric peptides. This could be attributed to the formation of incorrect disulfide bonds, which may cause a less compact structure than a correctly folded analog changing the elution properties. The third peak eluted in the same position as that of a peptide with a molecular weight similar to that of insulin or the native toxin. Activity measurements performed on fiddler crabs localized neurotoxic activity to this peak. Furthermore, Ellman's test (Ellman, 1959) was negative indicating that no free sulfhydryl groups were present. A fourth peak was

Table 4:2. Reoxidation of synthetic ShN I Analogs

	Air[a]	10:1 GSSG:GSH[b]	Dialysis Method[c]	Total Yield Pure Toxin[d]
	Yield of soluble material			
Tyr_1	7.0%	2.5%	76.6%	2.6%
$Nack_4$	4.8%	20.0%	100%	4.4%
Asn_6	7.0%	11.3%	100%	3.1%
Asn_7	9.6%	13.7%	96.7%	3.2%
Gln_8	11.9%	23.4%	100%	3.5%
Asn_{11}	10.8%	8.4%	78.2%	3.0%

[a]Reoxidation of disulfide bonds by air oxidation in 50 mM sodium acetate, pH 7.7 at 4° C for 96 h. Peptide concentration was 50 μg/ml.

[b]Reoxidation by the method of Ahmed et al. (1975).

[c]Reoxidation by the method of Sabatier et al. (1987).

[d]Total yield of correctly folded material following the purification described in the text.

detected on occasions where the preliminary desalting column had not removed all of the residual salts. Amino acid analysis of this fourth peak determined it to be residual glutathione. Reprocessing a portion of the material from the polymeric peak for the Tyr_1 analog using thioredoxin according to the method of Pigiet and Schuster (1986), resulted in approximately 15% additional monomeric material (Figure 4:3). Attempts to increase the amount of monomeric material by reprocessing each of the polymeric peaks for each of the analogs were not performed.

The monomeric peak obtained from the molecular sieving column was chromatographed on a phosphocellulose column. Gradient conditions were the same as used for isolating the synthetic native toxin (Pennington et al., 1987). As shown in Figure 4:4, further separation of a major peak with paralytic activity was achieved with this column. The activity peak from the phosphocellulose column was lyophilized and then subjected to reverse phase HPLC on C_{18} silica. Using a series of gradient as well as isocratic elution systems, the ShN I analogs were isolated in pure form (Figure 4:5). Ellman's test on each of the analogs was negative analysis. Characterization of the purified analogs also included amino acid analysis (Table 4:3).

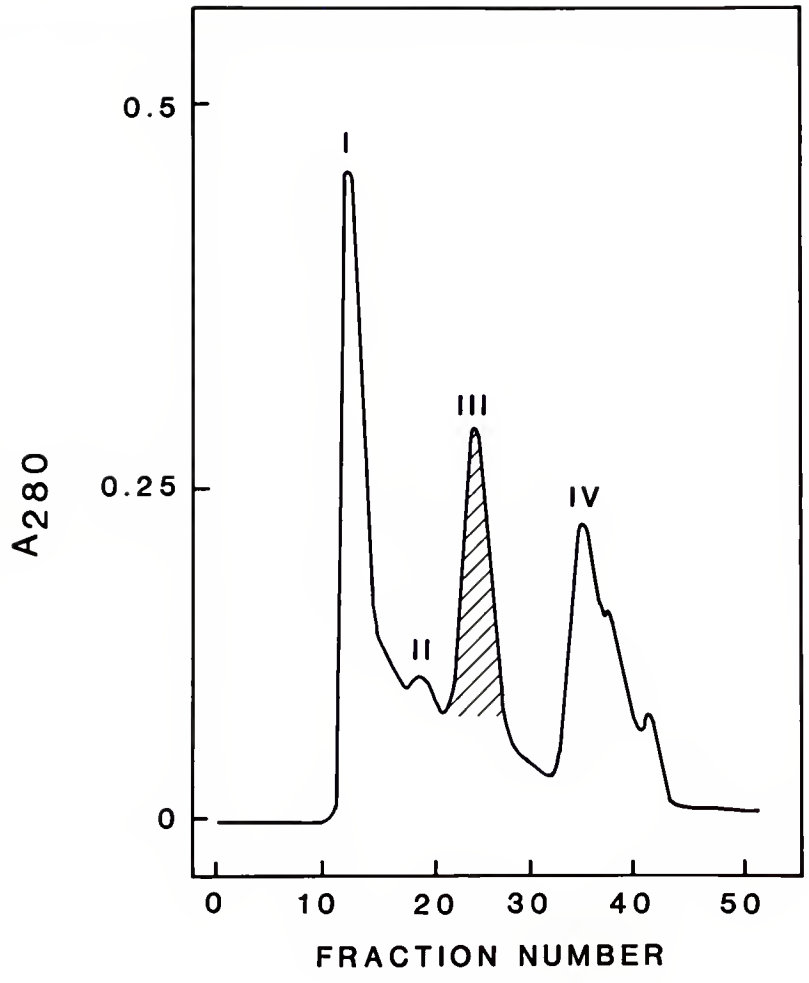


Figure 4:2. Gel Filtration Purification of Crude Refolded ShN I Analogs. Initial separation of the crude refolded analog was achieved with a Sephadex G-75 column (0.8 cm X 180 cm) equilibrated in 50 mM ammonium acetate. An active monomeric peak (hatched peak) was separated from the higher molecular weight polymeric fractions. (Shown here is $NacK_4$).

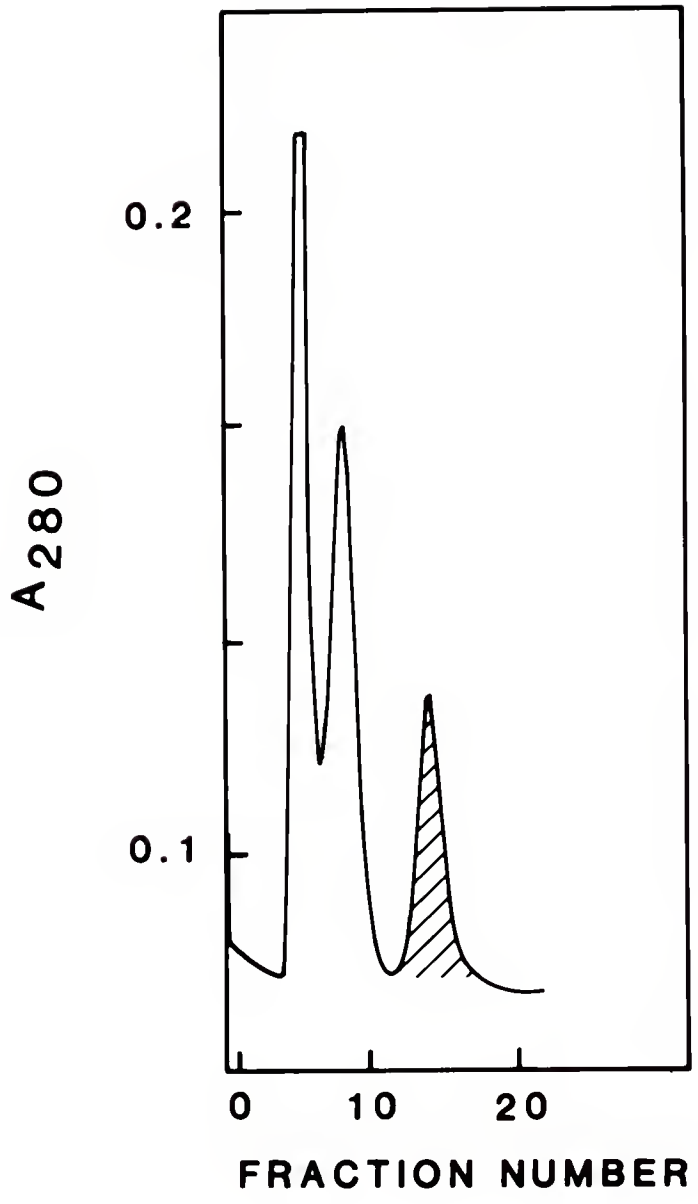


Figure 4:3. Separation of Active Monomer following Thioredoxin Reoxidation of Polymeric Peak. Separation of active monomer of Tyr_1 following thioredoxin mediated reoxidation was achieved on a Sephadex G-75 column (0.7 cm X 70 cm) equilibrated in 50 mM ammonium acetate. The first peak represented unreacted high molecular weight polymer, the second peak was thioredoxin, and the third peak (hatched peak) was monomeric Tyr_1 which was determined to be biologically active as described above.

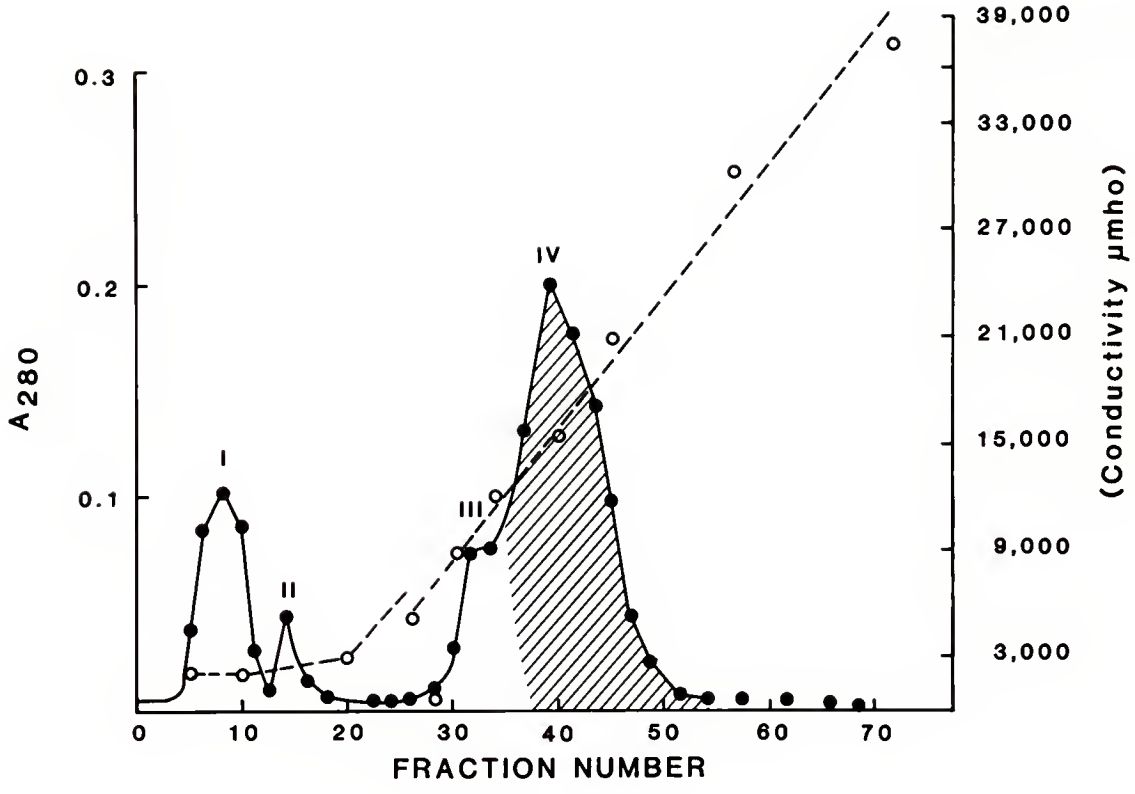


Figure 4:4. Phosphocellulose Purification of Analog Toxins. Monomeric refolded analogs were loaded on to a phosphocellulose ion exchange column equilibrated in 20 mM ammonium formate, pH 4.0. Peptides were eluted with a linear gradient from 20 mM to 500 mM ammonium formate, pH 4.0. Neurotoxic activity was isolated from the hatched peak IV. (Shown here is Gln_8).

Table 4:3: Amino Acid Analysis of Purified ShN I Analogs[a]

	ShN I	Tyr_1	$NacK_4$	Asn_6	Asn_7	Gln_8	Asn_{11}
Asp	6.0	5.86	6.04	5.89	5.85	6.11	5.93
Thr	4.0	3.86	3.78	3.83	3.95	3.88	3.78
Ser	2.0	1.97	1.84	1.89	1.85	1.82	1.80
Glu	2.0	1.95	2.10	2.01	1.91	1.96	2.02
Pro	2.0	2.00	2.14	2.11	2.10	2.08	1.96
Gly	4.0	4.28	4.09	4.19	4.11	4.13	4.16
Ala	6.0	5.00	6.00	6.00	6.00	6.00	6.00
Val	1.0	1.08	0.97	1.10	1.08	1.05	1.03
Ile	3.0	2.82	3.12	2.92	3.11	3.01	3.05
Leu	2.0	1.83	1.91	2.07	2.01	2.12	2.05
Tyr	2.0	2.78	1.88	1.92	1.85	1.83	1.86
Lys	5.0	4.85	5.08	5.22	4.88	5.07	5.19
Arg	2.0	1.90	1.98	2.12	2.12	1.94	2.07

[a]Hydrolysates from 6N HCl, 110° C, 24 h. Analysis by ninhydrin method. Cys and Trp values were not determined. All results based upon Ala.

Circular Dichroism. The CD spectra of the six synthetic ShN I analogs were characteristic of the native toxin structure in 0.2 M Na_2HPO_4, pH 6.8 buffer (Figure 4:6). All analogs showed similar ellipticity minima at 198 nm. Similarly, the shoulder region at 215-220 nm was also observed in each case. Analysis of the CD spectra for the RCM-ShN I derivative lacked these structural elements. Furthermore, CD structural analysis of one of the inactive peaks isolated from the phosphocellulose column also lacked these structural elements. These results indicate that the analogs have similar secondary structural elements as that of the native ShN I.

Fluorescence Emission. All of the analogs contained the single tryptophan residue located at position 30 of the native toxin. This residue has been determined to be exposed to the aqueous environment in the native toxin (Chapter III of this dissertation; Norton et al., 1988). The fluorescence emission spectra of the synthetic analogs have the same emission maxima as the native toxin (Figure 4:7). These results suggest that the structures of each of the analogs are similar to that of ShN I in that the tryptophan residue experiences the same environment.

Paralytic Activity. The synthetic ShN I analogs were tested for paralytic activity on fiddler crabs which has been used as our standard assay for neurotoxic properties. The LD_{50} values

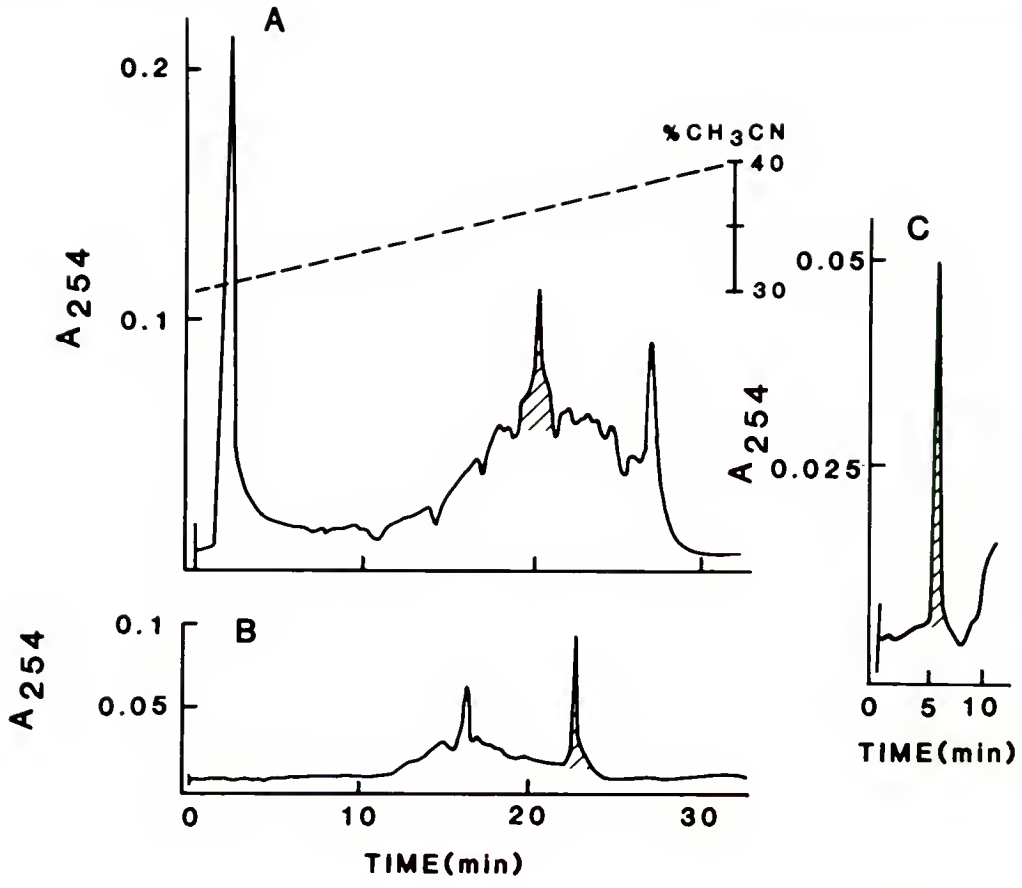


Figure 4:5. Reverse-phase HPLC Purification of Analog Toxins. A, Separation of the active peak (or major peak in the case of analogs with low activity) using an acetonitrile gradient from 30% to 40% into 0.1% TFA, in 30 min. B, Isocratic elution (33% acetonitrile into 0.1% TFA) of isolated active peak from gradient separation. C, Final separation of analog toxin using 35% acetonitrile isocratic elution. (Shown here is $NacK_4$).

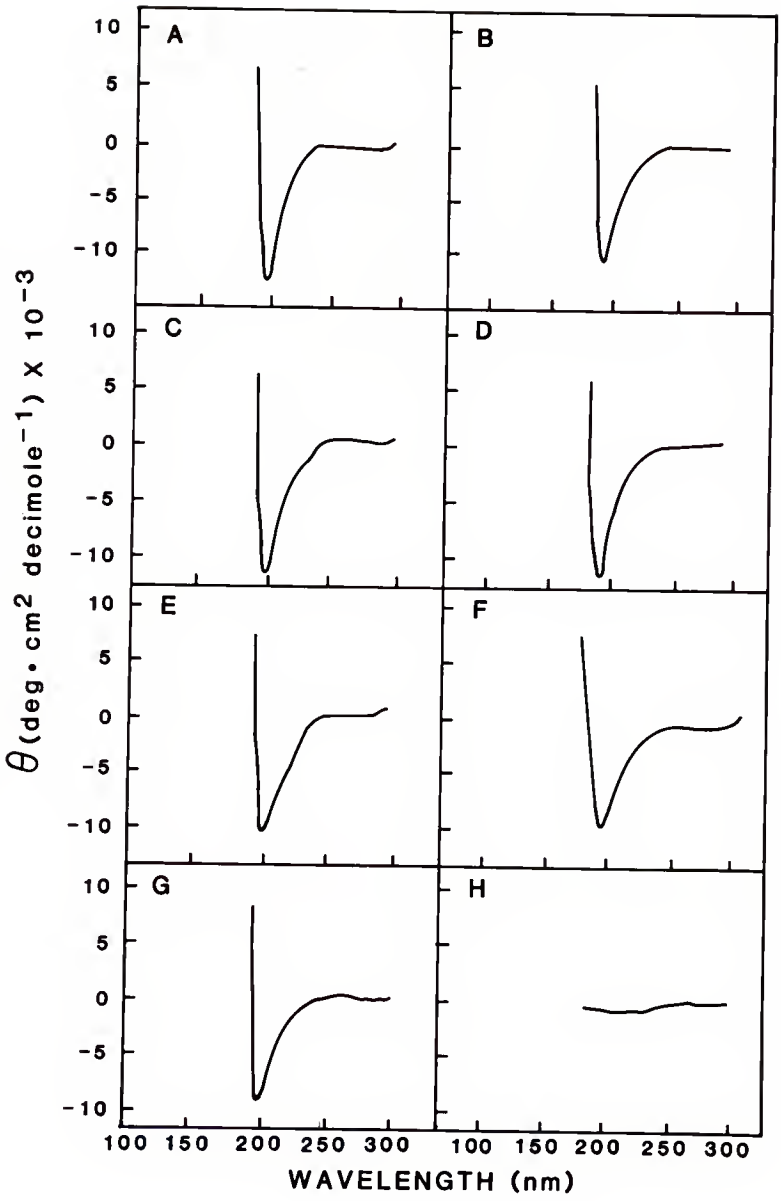


Figure 4:6. Circular Dichroism Spectra of Purified Analog Toxins. All recordings made with sample dissolved in 0.2 M Na_2HPO_4, pH 6.8. A, Native ShN I. B, Tyr I. C, $NacK_4$. D, Asn_6. E, Asn_7. F, Gln_8. G, Asn_{11}. H. RCM-ShN I, reduced and carboxymethylated derivative of the native toxin.

for each of the analogs are summarized in Table 4:4. These results implicate the tri-anionic region which is composed of the sequence Asp_6-Asp_7-Glu_8 as essential for activity. The Tyr_1 substitution possesses activity similar to that of the native toxin. The other substitutions $NacK_4$ and Asn_{11} have reduced activities by factors greater than 100. No measurements were determined for mammalian activity due to the lack of activity which the native toxin exhibits.

Binding Activity to Blue Crab Axolemma Vesicles. Competition binding studies with each of the synthetic analogs were performed on axolemma vesicles prepared from the walking leg nerve of blue crabs. Previous experiments have determined the K_D value of [^{125}I]-ShN I for this preparation to be 14 nM. The results of the competition assays for each of the analogs are shown in Figure 4:8. These results show that the Tyr_1 analog has $K_{0.5}$ of 50 nM. Every other analog has markedly increased $K_{0.5}$ values ($NacK_4$ and Asn_{11}) or was unable to displace [^{125}I]-ShN I (Asn_6, Asn_7 and Gln_8).

Discussion

The purpose of this work was to investigate some aspects of the structure-function relationships in ShN I. Our investigation was targeted at the N-terminal region of ShN I. We selected this region because of: (i) the high density of

Table IV: LD_{50}[a] Values of ShN I and Synthetic Analogs on Crabs[a]

Analog	LD_{50} (μg/kg crab wet weight)
ShN I, natural	2.92
ShN I, synthetic	3.14
Tyr_1	3.51
$NacK_4$	250
Asn_6	46,000
Asn_7	>100,000
Gln_8	>100,000
Asn_{11}	375

[a]*Determined by intrahemocoelic injection of fiddler crabs.*

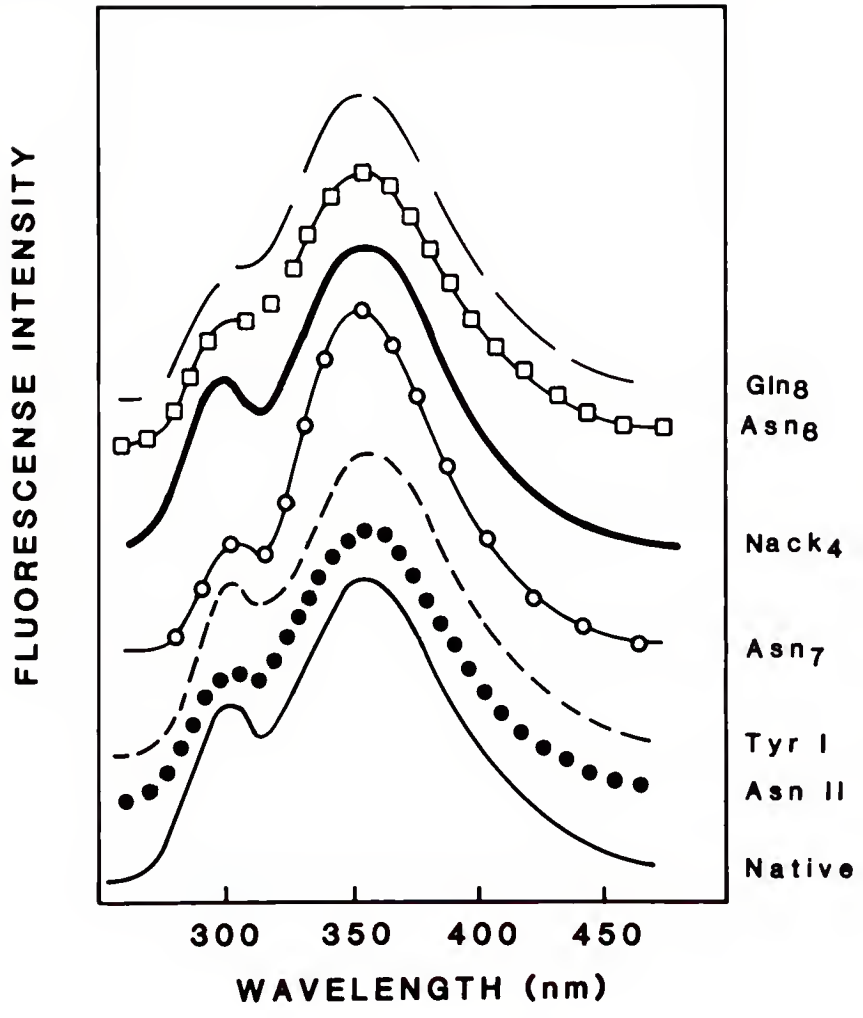


Figure 4:7. Fluorescence Emission Spectra for Analog Toxins. Emission spectra of analogs dissolved in 0.2 M Na_2HPO_4, pH 6.8. Each spectrum has been offset from 0 in order to simplify comparison of the results for each analog.

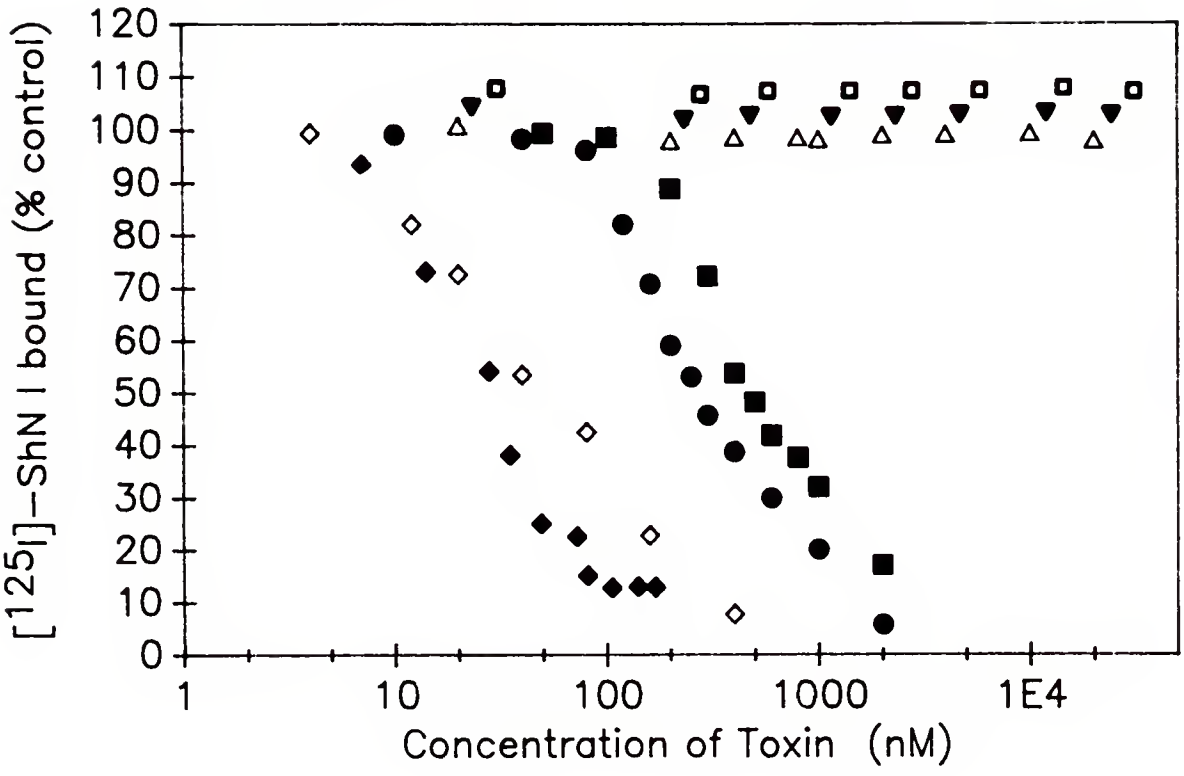


Figure 4:8. Inhibition of Specific [^{125}I]-ShN I binding by unlabeled Analog Toxins to Crab Axolemma Vesicles. Binding was measured under equilibrium conditions in the presence of the indicated concentrations of (◆) native ShN I, (◇) Tyr_1, (●) $NacK_4$, (△) Asn_6, (▼) Asn_7, (◘) Gln_8, or (■) Asn_{11}. Nonspecific binding in the presence of 10 μM unlabeled ShN I was subtracted out. Points falling above the 100% total binding for Asn_7 and Gln_8 have been offset in order to distinguish them. The values determined for these two analogs all fell within the range of ± 3% of 100% total [^{125}I]-ShN I bound causing these points to be clustered on top of each other.

charged residues, (ii) the conservation of the anionic cluster in both types of anemone toxins and (iii) inability to selectively chemically modify these anionic residues. Selection of the N-terminal Tyr_1 substitution was initially planned as an alternative site for iodination. From the activity studies and competition binding assays, several important conclusions can be drawn.

First, the clustered acidic residues, Asp_6-Asp_7-Glu_8, are essential for paralytic activity in fiddler crabs. These residues are invariant in the type 2 anemone toxins. An anionic site (Asp_7 and Asp_9) is in a similar position in the most of the type 1 toxins as well. Furthermore, replacement of any one of these residues with the neutral isostere abolishes the ability of these analogs to competitively displace the radiolabeled [^{125}I]-ShN I from axolemma vesicles. Structurally, each of these analogs resembles the native toxin (Figures 4:5 and 4:6). The similarity that the synthetic native ShN I (Pennington et al., 1988) as well as the Tyr_1 analog show to the native toxin in both structure and activity are strong evidence that the synthetic molecules are biologically functional. This suggests that the lack of activity and destruction of binding properties of each of these analogs (Asn_6, Asn_7 and Gln_8) results from the replacement of any one of these anionic residues. Proton NMR studies on Hp II have determined that this anionic cluster falls within the loop structure of residues 6 through 17, which has

been proposed to be essential for activity (Wemmer et al., 1986).

Another invariant residue in type 2 anemone toxins is Lys_4. This residue is positioned between two cysteine residues which form disulfide bonds in the molecule. This particular region has been determined to exist as part of the β-sheet structure in Hp II (Wemmer et al., 1986). Replacement of the ϵ-amino group of Lys_4 with the acetylated derivative reduced the activity of this toxin by nearly 100 fold. The binding activity was also reduced to a lesser extent (Figure 8). These data are less conclusive than the case for the tri-anionic region, but they suggest the involvement of this residue in the receptor binding domain.

The position of Asp_{11} is one of the residues which differ between ShN I and Hm III, another type 2 anemone toxin. Hm III exhibits just the opposite species selectivity of ShN I, it has the highest reported mammalian toxicity of any anemone toxin (Zykova et al., 1985). These two toxins have highly homologous sequences differing at only six positions. The Asn_{11} analog probed the importance of this negative charge. The binding properties of this analog indicate that it is also important in recognition of the receptor. The position of this residue is also in the loop structure which was reported for Hp II (Wemmer et al., 1986). The residue at position 11 in Hm III is tyrosine. Tyrosine is capable of ionizing to the negatively charged phenolate group at much more alkaline pH values than

required for ionization of carboxylate groups (Keim et al., 1973). Thus, a direct correlation to the species selectivity differences that this particular residue may confer cannot be argued solely as an ionic interaction. The possibility exists for the combination of aromatic, ionic, or hydrogen bonding properties to be involved.

Previous structure function studies on type 1 sea anemone toxins have been rather controversial. Barhanin et al. (1981) reported that chemical modification of Arg_{14} abolished activity of As II. Kolkenbrock et al. (1983) did not observe this effect when chemically modifying As II with 1,4 cyclohexanedione; nor did phenylglyoxal treatment of Ax I abolish activity (Newcomb et al., 1980). Modification of Arg_{13} in Hm I, a type 2 anemone toxin, resulted in 20 fold decrease in activity (Kozlovskaya et al., 1982). One of the most interesting reports was that of modification of the three carboxyl groups in As II. This modification resulted in a derivative which was biologically inactive yet it functioned as a competitive antagonist (Barhanin et al., 1981). These results could not be confirmed by Kolkenbrock et al. (1983). Norton and Gruen (1984) reported that chemical modification of Ax I carboxyl groups abolished the activity. Amino group chemical modification has been reported for toxins of both type 1 As II (Barhanin et al., 1981) and type 2 Hm I (Kozlovskaya et al., 1982). In both cases, significant reduction of biological activity was reported. Although most of the chemical modification data reported is for type 1 anemone

toxins, it is important to realize that many of the residues which were being studied are conserved between the two classes.

In light of the problems associated with chemical modification, namely residual unmodified protein and/or lack of selectivity, it is difficult to assess the importance of any particular residue. Chemical synthesis of analog structures has been used recently in several different systems to investigate particular residues (Andreu et al., 1985; Moe and Kaiser, 1985; Lu et al., 1987). The advantage to chemical synthesis over modern genetic methods is the ability to introduce nonbiological amino acid residues (for example the $NacK_4$ analog), and/or the incorporation of isotopically enriched amino acids at specific positions within the peptide chain. Use of these types of techniques can provide data which will eliminate several of the problems associated with chemical modification studies that have attempted to characterize residues which are important for binding to the Na channel.

The distinction between two different anemone receptors on the Na channel has only recently been reported (Schweitz et al., 1985; Chapter II, this dissertation). As the major structural elements of both types of anemone toxins appear to be very similar (Gooley and Norton, 1986; Wemmer et al., 1986: Widmer et al., 1987) the differences between them must lie within the nonconserved sequences. A particularly good example of this structural similarity is between two different scorpion toxins: the small insect toxin, I_5A, (35-residue toxin) (Arseniev et

al., 1984) isolated from *Buthus eupeus* and the long mammalian scorpion toxin CsE III (65-residue toxin) (Fontecilla-Camps et al., 1980; Almassy et al., 1983) isolated from *Centruroides sculpturatus Ewing*. The structures of these two toxins are remarkably similar despite the differences in the primary structures, yet they display completely different mechanisms of action.

There is no reason to believe that the entire ShN I molecule is involved in binding to the type 2 anemone toxin receptor present on the Na channel surface. It seems more likely that certain parts of the molecule would form some type of recognition site, while other portions of the molecule are important for maintaining the structure of this site. Our results indicate that the invariant tri-anionic cluster is most likely a required recognition site of the type 2 toxins. Other residues outside of this region may also be important to this site, but they may also be involved in interactions with the receptor surface in different animals such as position 11 in ShN I. From an evolutionary standpoint, all anemone toxins may have originated from a common ancestral gene. Natural selection pressures forced the production of toxins with selective (selective toward only mammals or crustaceans) or combined activities (nearly equivalent activities on both animal types). Probing the interactions at each receptor site may eventually be used for the development of very selective pesticides or drugs which would be only targeted to a particular tissue or animal.

CHAPTER V

CONCLUSIONS AND DIRECTIONS

This work has resulted in the characterization of a new sea anemone binding site located on the Na channel. It is extremely interesting that the K_D for ShN I, to this receptor in crustaceans is nearly 1000 times lower than that measured for mammalian species. Binding to this receptor does not appear to be influenced by membrane potential or veratridine. In competition studies with As II, a type I anemone toxin, [^{125}I]-ShN I was not displaced from its receptor site. Finally, ShN I was unable to displace the radiolabeled α-scorpion toxin AaH II from rat brain synaptosomes. Thus, the receptor site appears to be unique to the ShN I and possibly Hp II another type 2 anemone toxin (Schweitz et al., 1985). It is reasonable to expect there to be more toxin binding sites on the Na channel. The size of the Na channel, as well as its central involvement in electrical processes, make it the ideal target for a variety of different molecules.

Using solid-phase peptide synthesis, we have succeeded in making six monosubstituted analogs of ShN I to further probe the interactions that this polypeptide toxin has with this receptor. The results of these studies allowed us to conclude that the tri-anionic site located at positions 6 through 8 in the

sequence is essential for activity *in vivo* and binding to the receptor *in vitro*. Substitutions at positions 4 and 11 affected both the biological properties as well as increasing the relative $K_{0.5}$ by a factor of 50. Substitution of the N-terminal residue with tyrosine showed very similar properties in both *in vitro* as well as *in vivo* systems.

From the analog studies, it appears that the ShN I molecule associates with a receptor site with an absolute requirement for this cluster of negative residues. With the elucidation of the Na channel sequence (Noda et al., 1984; Noda et al., 1986; Salkoff et al., 1987), one may predict a site which would bind ShN I, may have a clustering of basic residues on an extracellular region of the Na channel. A very interesting experiment would be to attempt to covalently label the Na channel with [^{125}I]-ShN I, digest the complex and isolate the labeled peptides. Sequence analysis of the labeled peptide would then give an even more detailed picture of the receptor site. One would expect some type of polycationic site to interact with the reciprocal site of ShN I.

Several other very interesting analogs could also be constructed to identify which residues confer the species selectivity to ShN I versus Hm III. By systematically interchanging residues at each of the six nonconservative positions between ShN I and Hm III, one would expect to generate analogs which would begin to have properties of both molecules. For example, the substitution at position 39 replaces an

isoleucine residue in ShN I with a proline residue in Hm III. The position of this proline residue in the sequence places it immediately before the antiparallel β-sheet structure (Gooley et al., 1986; Wemmer et al., 1986; Widmar et al., 1987). Proline positioning generally confers a kink or bend in the structure of a protein or peptide. Such a substitution may generate an analog with higher mammalian activity than that of ShN I due to its slightly different structural properties resulting from the kink introduced by the proline.

In conclusion, this work shows the utility of solid-phase peptide synthesis with elucidating the macromolecular binding properties of Na channel. In particular, we have identified a novel receptor site on the Na channel and identified several critical residues which participate in binding to this site through the use of state-of-the-art technologies.

APPENDIX

DISULFIDE BOND FORMATION IN PROTEINS

Disulfide bond formation in proteins is a post-translational modification which is dependant on the conformation of the protein. For two cysteine residues to form a disulfide bond, the two α-carbons must lie within 4-9 Å of each other, and the adjoining peptide backbone must also be in the right orientation to allow the five bond rotations to position the side-chain favorably (Richardson, 1981). The covalent nature of the bond formed between the two sulfur atoms contributes to the stabilization of the structure of the protein molecule (Anfinson and Sheraga, 1975; Thorton, J.M., 1981). However, this covalent bond can be broken by adding an excess of a reducing agent such as dithiothreitol or β-mercaptoethanol. Upon removal of the reducing agent, the protein disulfide bonds are broken and the protein has two free sulfhydryl groups for every one of the disulfide bonds broken. Reoxidation of the disulfide bonds is possible, but this problem is much more complex than simply breaking them.

Disulfide bond formation requires an electron acceptor molecule to be present. Classical air oxidation of disulfide bonds relies on O_2 in the presence of metal ions such as Cu^{2+}

which participate in the reaction by transiently binding to the thiol group in the process of generating the thiolate. The formation of the disulfide bond results from the nucleophilic attack of the thiolate group on a thiol group in close proximity (Creighton, 1984).

Glutathione, due to its high concentration in most organisms as well as its favorable redox potential (E_o'= -0.34 V), is believed to be the electron donor/acceptor system used in vivo. Glutathione can exist as either the oxidized form GSSG or the reduced form GSH. Glutathione interacts with thiols and disulfides in the thiol-disulfide exchange reaction by the following scheme:

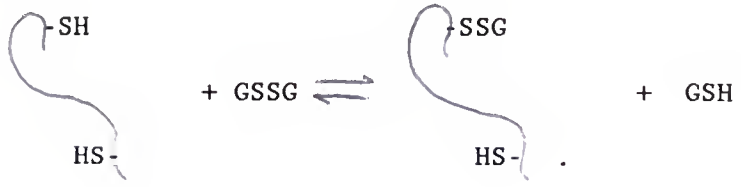
A. GSH + RSSR $\rightleftharpoons$ GSSR + RSH

B. GSH + GSSR $\rightleftharpoons$ GSSG + RSH.

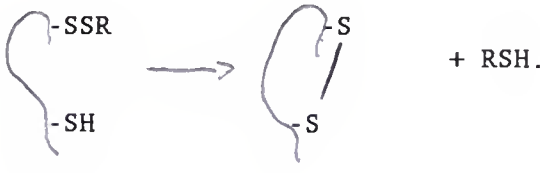
This reaction is rapid at alkaline pH due to the deprotonation of the thiol group (pK_a value 8.2). The rate of the reaction is dependent upon concentrations of the reactants, temperature, pH, ionic strength, as well as certain properties associated with the disulfide such as energetically strained bonds (Creighton, 1984).

The process of forming a disulfide bond in proteins, following the addition of glutathione, takes place in two sequential thiol-disulfide exchange reactions. The first

reaction is the reaction of the cysteine residue of a protein with GSSG to form the mixed disulfide shown in the following reaction:



This reaction is bimolecular in both directions. Thus, the rates of the reaction are dependent upon the concentrations of the reduced and oxidized glutathione. The second step is formation of the protein disulfide bond when a free sulfhydryl group of the protein in close proximity reacts with the mixed disulfide



Following the initial formation of disulfide bonds, the protein may then go through successive rounds of disulfide interchange until the most energetically stable conformation is attained. Certain proteins such as thioredoxin (Pigiet and Schuster, 1986) and liver microsomal protein disulfide isomerase (Creighton et al., 1980) have been shown to catalytically enhance this process when in the proper redox system. Each of these enzymes have homologous catalytic domains containing a disulfide bond that protrudes out from

the protein surface (Edman et al., 1985). The reactivity of these proteins is believed to be due to the abnormally low pK_a of one of the cysteines in this loop (Kallis and Holmgren, 1980). This allows for greater reactivity at physiological pH values.

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BIOGRAPHICAL SKETCH

Michael Pennington was born in Charlotte, North Carolina, on March 9, 1962. He lived in Charlotte throughout his first eighteen years. Following the completion of his high school education at South Mecklenberg High School in Pineville, he began his college education at the University of North Carolina at Chapel Hill. During his sophomore year at the university, he was hired as a technician in Dr. Richard G. Hiskey's laboratory. This laboratory was geared toward the biophysical study of the blood-clotting protein prothrombin. Part of his responsibilities involved a weekly trip to the local slaughterhouse, where he would begin the laborious task of prothrombin isolation. Having succeeded in purifying this protein, he then would activate this protein from the zymogen form and purify the activation fragments for graduate student research projects. His interest in the physical aspects of biochemistry carried through his senior year when he geared his curriculum toward enzymology and protein chemistry.

In the fall of 1984, Michael began his graduate studies in the Department of Biochemistry and Molecular Biology at the University of Florida in Gainesville. He developed an immediate interest in the study of the Na channel after completing a short

rotation project in Dr. Kimon Angelides's laboratory working with Thomas J. Nutter. Using the knowledge gained from this rotation, Michael entered into Dr. Ben M. Dunn's laboratory for a rotation. Fortuitously, Dr. Dunn had entered into a collaborative project with Dr. William R. Kem in the Department of Pharmacology and Therapeutics. The project involved the investigation of structure-function relationships in sea anemone neurotoxins. This project was able to pull several of Michael's interests together, namely organic synthesis, Na channel research, and SCUBA diving in the Florida Keys. Michael remained in Dr. Dunn's laboratory learning many new techniques.

Michael's extracurricular activities included playing lead guitar for one year in the popular college band Cairo, breeding Afghan hounds, and sky diving. For one semester, he was the president of the Falling Gators sky diving club.

After finishing his Ph.D., Michael will move to Bloomfield, New Jersey, to begin working for Schering-Plough Research Corporation. He will direct a peptide synthesis laboratory geared toward identifying the functional regions of several different lymphokines. This position offers Michael the opportunity to extend his education in peptide chemistry and receptor binding interactions.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ben M. Dunn
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel L. Purich
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas W. O'Brien
Professor of Biochemistry and
Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William R. Kem
Associate Professor of
Pharmacology and Therapeutics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Alfred F. Esser
Professor of Experimental Pathology
and Biochemistry and Molecular
Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1988

Dean, College of Medicine

Dean, Graduate School